

1969

Bovine virus diarrhea virus-host cell interaction

Ricardo Francisco Rosenbusch
Iowa State University

Follow this and additional works at: <https://lib.dr.iastate.edu/rtd>



Part of the [Microbiology Commons](#)

Recommended Citation

Rosenbusch, Ricardo Francisco, "Bovine virus diarrhea virus-host cell interaction " (1969). *Retrospective Theses and Dissertations*. 3771.
<https://lib.dr.iastate.edu/rtd/3771>

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

This dissertation has been
microfilmed exactly as received 70-7731

ROSENBUSCH, D.V.M., Ricardo Francisco, 1942-
BOVINE VIRUS DIARRHEA VIRUS-HOST CELL
INTERACTION.

Iowa State University, Ph.D., 1969
Microbiology

University Microfilms, Inc., Ann Arbor, Michigan

BOVINE VIRUS DIARRHEA VIRUS-HOST CELL INTERACTION

by

Ricardo Francisco Rosenbusch, M.D.

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Veterinary Microbiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Ames, Iowa

1969

TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	3
Viral Interference	3
Interference Mediated by Interferon	6
Interference Between Strains of Bovine Virus Diarrhea	8
Characterization and Classification of BVD Virus	10
Intracellular Steps in BVD Virus Replication	11
Viral Induced Cell Death	15
MATERIALS AND METHODS	19
Cell Cultures	19
Virus Strains	20
Infectivity Assays	21
Characterization of Virus Strains	22
Assay for Interference	23
Separation of Infectivity from Interfering Activity	24
Virus Purification	26
Virus Growth Curves	26
Effect of Actinomycin D on C24V Virus Growth	28
Metabolic Inhibitions	30
Leakage of Proteins	32
Inhibition of Protein Leakage	33

	Page
RESULTS	34
Characterization of Virus Strains	34
Separation of Infectivity from Interfering Activity	36
Association of Interference with Infectivity	44
Virus Growth Curves	46
Growth of Virus in the Presence of Actinomycin D	52
Site of RNA Synthesis	53
Alterations in Metabolism of Infected Cells	53
Virus-induced Protein Leakage	57
DISCUSSION	67
SUMMARY	86
BIBLIOGRAPHY	89
ACKNOWLEDGMENTS	100

INTRODUCTION

The virus of bovine virus diarrhea (BVD), has been the subject of extensive studies in recent years. Knowledge gained from these studies has provided insight into the pathogenesis of the disease and has made possible the rapid diagnosis and prevention of bovine virus diarrhea. In order to progress further with effective control and eventual eradication of this disease, an increased understanding of the pathogenesis, the occurrence of viral latency and the epidemiology of BVD is necessary. Before such studies can be carried out successfully, it is imperative that additional information be available concerning the intracellular mechanisms involved in virus-host cell relationships.

Quite apart from the disease standpoint, any basic data obtained on the interactions between BVD virus and its host cell might also apply to other viruses that share antigenic components, physicochemical characteristics, or both. The virus of hog cholera is considered to be similar to BVD and the viruses of equine arteritis and human serum hepatitis may also be related. Availability of such basic information might aid in placing these viruses within the current virus classification scheme, either as members of a presently defined group or in a separate category.

There is abundant information available at the molecular level for a limited number of "type" viruses from some groups

in the classification scheme. These "type" viruses have been utilized to study various biochemical events in mammalian host cells and have furnished information not easily obtained by other means. In the case of BVD virus, inadequate knowledge about the virus precludes its use in this way.

The objectives of the present study were to confirm and extend data previously reported by other authors in three main areas of interest: 1) the interference produced by noncytopathogenic BVD strains upon superinfecting cytopathogenic BVD strains, 2) characteristics of the replication of BVD virus such as the intracellular site of synthesis of viral components and the time at which these products are formed, and 3) the mechanism involved in the production of cytopathology by a strain of the virus that is capable of killing its host cell.

LITERATURE REVIEW

Viral Interference

One of the ways that a virus interacts with host cells is to inhibit the multiplication of superinfecting viruses. The interfering virus can exert its action either on certain host-cell structures making them unavailable to the superinfecting virus or can directly interfere with the replication of the superinfecting virus. Observations on the occurrence of interference between many viruses and excellent reviews of the subject are included in articles by Schlesinger (85) and Wagner (98).

The basic mechanisms of viral interference have not been fully elucidated but four different models have been described in detail. High multiplicities of infection with certain viruses can block the specific cell receptors needed by a closely related superinfecting virus. Baluda (10) observed this phenomenon between ultra-violet irradiated Newcastle disease virus and the intact virus. Steck and Rubin (91) found that this type of interference occurred between avian leukosis virus and its homologous Rous sarcoma virus pseudotypes. Similar observations were made by Crowell (22) on antigenically related strains of Cocksackie virus.

Not only can viruses compete for receptors located on the cell surface but also for intracellular sites. In this model, interference between viruses is usually reciprocal and is only

established after the interfering virus has penetrated into the cell and started to synthesize viral components. A very high multiplicity of interfering virus is required if the challenge virus is added simultaneously. However, infections with equal multiplicities of both viruses may give rise to interference if the interfering virus is added at least one hour before challenge. The nature of the intracellular sites of interference has not been clearly demonstrated and may vary with the system. Dubes and Rouhandeh (27) observed this type of interference between infectious RNA of two strains of poliovirus while Cords and Holland (21) detected it between enterovirus mutants differing in their sensitivity to guanidine. Zebrovitz and Brown (106) suggested that such a mechanism was operating in the interference between temperature-sensitive mutants of Venezuelan and Eastern equine encephalomyelitis viruses.

A virus can carry within its genome the information necessary to control or interfere with the synthesis of its own components. The classical model of a repressor gene controlling the expression of genetic information was described by Jacob and Monod (60), and has been successfully used to explain the phenomenon of prophage immunity (59). This is an interference phenomenon observed in bacteria infected with temperate DNA bacteriophages. In this model, the interfering virus synthesizes a repressor protein that is capable of binding to the incoming genome of the challenge virus to block its transcription.

Robertson et al. (79) have reported that the structural protein produced by an RNA bacteriophage acts as a repressor since it prevents the transcription of viral genomes. The observations of Huang and Wagner (55) on the autointerference of vesicular stomatitis virus by defective T particles may be an example of such a phenomenon with animal viruses. The authors observed that a requirement for interference was the introduction of the RNA fragment of the T particles into the cell and the synthesis of protein. On the basis of the time at which maximal interference was expressed, they suggested that the T particles induced the synthesis of structural proteins. These proteins attached to the challenge virus genome and prevented it from synthesizing the nonstructural proteins necessary for successful replication.

Since the formation of specific viral complexes is necessary during the replication cycle of viruses (i.e. nucleic acid - polymerase, nucleic acid core - structural proteins, etc.), it would be expected that abnormal and perhaps nonfunctional complexes arise during mixed infections. Roizman (80) observed interference between two mutants of herpes simplex virus. A mutant that could replicate its genome but was unable to synthesize structural proteins interfered with the multiplication of another mutant that produced structural proteins but had decreased rates of genome synthesis. Interference resulted from the complexing of structural proteins of the challenge

mutant with DNA of the interfering mutant to form nonfunctional complexes. A similar mechanism may be involved in the phenomenon of intrinsic interference described by Marcus and Carver (68). According to these workers, a number of RNA viruses are capable of inducing interference specific against Newcastle disease virus strains. Zuckerbraun and Marcus (107) have more recently observed that this interference is due to the expression of 3 early genes of the interfering virus, possibly those associated with the viral polymerase activity. These proteins may bind to the challenge Newcastle disease virus genome and form a nonfunctional complex.

Interference Mediated by Interferon

Isaacs and Lindenmann (56) first described a virus-interfering factor produced in cells from chorio-allantoic membranes exposed to heat-inactivated influenza virus. This factor, when added to normal chorio-allantoic membrane cells, prevented the multiplication of influenza virus. This material, which was termed interferon, was a pH 1 resistant protein (64), was produced by the host cells (18) and only protected cells from the same animal species (56). Subsequent work has indicated that interferon is produced by cells in response to the introduction of foreign polyanions (58, 92).

Heller (51) was the first to observe that the cell genome participated actively in the production of interferon. Taylor

(95), Friedmann and Sonnabend (39), and Lockart (65) reported that cell-directed RNA and protein synthesis were necessary for the expression of interferon action. Later, Marcus and Salb (67) described a model involving two cellular genes responsible for the interference mediated by interferon. One gene is activated by foreign polyanions and directs the synthesis of interferon. The primary function of this protein is to induce a second gene in the same or other cells to direct the synthesis of a translation-inhibitory protein. This protein associates with cellular ribosomes and permits the translation of cellular RNA while blocking the translation of a challenge virus genome. In order to exert its action, interferon has to be cell-specific and readily transportable through cellular membranes (18).

Numerous observations have been made on the heterogeneity of interferon sizes, as reported in reviews by Ho et al. (53) and Fantes (28). The classical description of interferon specifies a protein with a small molecular weight (approximately 20,000 to 40,000). However, proteins with interferon activity and much larger molecular weights are produced in response to certain viruses and to most nonviral inducers. Hallum et al. (50) observed an interferon of a molecular weight of 89,000 daltons in the serum of mice injected with endotoxin. Smith and Wagner (88) obtained interferons of more than 134,000 in molecular weight from rabbit cells stimulated by virus or

endotoxin and Fournier et al. (37) described a human interferon of a molecular weight of 160,000.

Bacterial endotoxins are effective inducers of interferon. As pointed out by Wagner and Smith (99), these products are sometimes present in cell culture media and can affect the results of interferon assays. Allison et al. (3) reported that interferon production by cell cultures was a very sensitive test for the presence of pyrogens in the water used for media preparation.

Fantes included in his review (28) a discussion of the properties of several bovine interferons. These materials were resistant to pH 2 dialysis and to treatment with heat or ether, but were unstable when subjected to freezing and thawing. Size determinations indicated a heterogeneous mixture ranging from 30,000 to 60,000 in molecular weight.

Interference Between Strains of Bovine Virus Diarrhea

The first report of interference with a cytopathogenic strain by noncytopathogenic strains of BVD virus was that of Gillespie et al. (43). They observed that cultures preinfected for at least 3 days with a number of different noncytopathogenic strains became resistant to a plaque-forming cytopathogenic strain. By using this procedure they were able to titrate the noncytopathogenic strains in cell cultures and perform neutralization tests.

A slight and transient interference against vesicular stomatitis virus was observed in cultures infected with non-cytopathogenic strains by Kniazeff and McClain (62) and Gratzek (45). Malmquist et al. (66) reported that hog cholera virus-infected cell cultures were resistant to a cytopathogenic BVD strain. These authors observed that the superinfecting BVD virus was not able to synthesize infectious virus or soluble antigen in these cells. Since interfering activity in hog cholera virus preparations could not resist pH 2 dialysis, they concluded that the interference was due to other factors than interferon. Strains of BVD virus are capable of suppressing interferon production by cell cultures treated with interferon-inducing viruses. Diderholm and Dinter et al. (25) reported that a noncytopathogenic as well as a cytopathogenic strain of BVD suppressed the induction of interferon by Newcastle disease virus. However, their observations indicated that BVD virus is sensitive to interferon. These authors also reported that cultures infected with a noncytopathogenic BVD strain were resistant to a cytopathogenic BVD strain but completely susceptible to heterologous viruses such as pseudorabies, foot and mouth disease, a bovine enterovirus and Newcastle disease virus. The results led them to conclude that the interference phenomenon was not mediated by interferon and that, in fact, BVD virus was unable to induce interferon synthesis. Singh (86) confirmed that a noncytopathogenic BVD strain interfered with cytopathogenic BVD viruses but had no effect on unrelated viruses.

Characterization and Classification of BVD Virus

In a recent classification scheme, Wilner (105) has placed BVD viruses among the "RNA-helical-enveloped viruses." Bürki (19) has proposed their inclusion in a new group designated "hemoviruses" which would include the viruses of BVD, hog cholera, equine arteritis and possibly human serum hepatitis.

Hermódsson and Dinter (52) reported that BVD virus was ether sensitive and grew in the presence of iododeoxyuridine. Filtration and electron microscopy indicated a particle size of 40 nm. Dinter (26) concluded that hog cholera viruses shared these properties with BVD. Also, both viruses were somewhat resistant to trypsin digestion. Gutekunst (47) observed that these properties also characterized a new cytopathogenic BVD isolate.

Hafez et al. (49) gave the size of BVD virus as between 46 and 60 nm depending on the method of preparation for electron microscope observation. They also saw dense inner cores of 24 to 26 nm in diameter. Mayr et al. (69), Cunliffe (23) and Horzinek et al. (54) reported an outer diameter of 40 nm and a core of 28 nm by electron microscopy for hog cholera virus. The buoyant density of the virus was 1.15 to 1.16 g \times cm⁻³.

Tanaka et al. (94) and Gutekunst (47) were able to filter BVD virus through 50 nm filters while Fernelius (30) observed that the virus passed through 50 nm filters and in a few

instances through 25 nm filters.¹ In a later publication Fernelius (32) reported that the filtrability of BVD viruses was strongly influenced by freeze-thaw treatments or prolonged storage. Cytopathogenic strains became filtrable through 50 nm filters after prolonged storage while noncytopathogenic strains passed through 100 nm filters only after freeze-thaw treatments. He reported no infectivity that was filtrable through 25 nm pores. Fernelius also determined that the buoyant density of BVD virus is $1.15 \text{ g} \times \text{cm}^{-3}$ (31) and that the infectivity from fresh virus materials could be recovered in two distinct peaks from the eluates of agarose chromatography columns (33).

Intracellular Steps in BVD Virus Replication

Gratzek (45) reported that adsorption of cytopathogenic BVD virus was very rapid, being nearly completed in 30 minutes. He also observed that cell associated infectivity increased between 8 and 10 hours after infection and that virus was released 4 hours later. Gillespie et al. (44) noted increases of cell-associated and free virus at 10 to 12 hours after inoculation of cells with various cytopathogenic BVD strains. They also observed that the virus concentrated in the supernatant fluids and suggested that the complete infective particle is formed in the cytoplasm of the cell. With another cytopathogenic

¹The Millipore Filter Corporation has supplied these filters designating their pore size as $10 \pm 2 \text{ nm}$ in the past. Recent measurements give their pore size as $25 \pm 3 \text{ nm}$.

strain, increases of intracellular virus were noticed at 6 hours by Gutekunst (47) and extracellular virus was detectable at 9 hours.

Gutekunst and Malmquist (46) observed that BVD viruses produced a soluble antigen that was separable from the infectious particle. This antigen crossreacted with a similar antigen produced by hog cholera virus, as reported by Darbyshire (24). The soluble antigen of BVD virus was ether resistant, trypsin sensitive and pH 3 sensitive. It was produced at 3 hours after infection and was later incorporated into virions, according to Gutekunst and Malmquist (48).

Various investigators have used fluorescent antibody techniques to determine the intracellular sites of viral antigen production. Mengeling et al. (70) observed cytoplasmic fluorescence in BVD infected cells treated with specific fluorescent antibodies. In sequential studies, Fernelius (34) described an "early" nuclear followed by cytoplasmic fluorescence. With hog cholera virus, Mengeling et al. (71), Robertson et al. (78) and Aynaud (5) reported a distinct cytoplasmic fluorescence. In sequential studies, Solorzano (90) recognized a faint nuclear fluorescence that was followed by marked cytoplasmic localization.

Information on the intracellular site of viral genome synthesis can be valuable in studies of virus-host cell relationships. Such data is available for a number of

unrelated lipid enveloped-RNA viruses, but has not been obtained to date for BVD or hog cholera viruses. Scholtissek et al. (82) detected a change in the amount and type of RNA synthesized by the nucleus of cells following infection with fowl plague virus. They concluded that this represented RNA synthesis directed by the genome of the myxovirus. Wheelock (103) reported that Newcastle disease virus, a paramyxovirus, replicated its RNA in the cytoplasm. Bukrinskaya et al. (17) provided evidence that Newcastle disease virus replicated its genome in the nucleolus from where it was rapidly transported to the cytoplasm. Experiments utilizing long pulses of radioactive label gave results similar to those of Wheelock. In previous papers, Bukrinskaya and Zhdanov (15) and Bukrinskaya (16) had reported that a paramyxovirus increased the synthesis of RNA in the nucleolus while a myxovirus caused a corresponding increase in the extranucleolar part of the nucleus of actinomycin-treated cells. Ter Meulen and Love (96) saw intranuclear and cytoplasmic RNA inclusions in cells infected with a myxovirus, influenza. They reported that although the nuclear inclusions seemed to appear before those in the cytoplasm, some cells only had cytoplasmic inclusions. Recently, Skehel and Burke (87) obtained evidence that fowl plague virus replicates its RNA in the cytoplasm of the host cell. The authors also saw increases in nuclear RNA synthesis of infected cells which could have been virus-directed. Western equine encephalitis virus, an

arbovirus, replicated its RNA in cytoplasmic structures, according to Wecker and Richter (101). This observation was extended by Friedman and Berezesky (40) and Ben-Ishai et al. (12) to other arboviruses.

Experimentation concerned with the culture of viruses in the presence of actinomycin D, a transcriptase inhibitor, has led to a suggestion that sensitivity of viruses to this drug be used as a criterion of virus classification. A number of viruses were classified by Reich (77) on their ability to grow in the presence of various levels of actinomycin D. He grouped them as sensitive, intermediate or indifferent. He suggested that sensitive viruses, such as fowl plague virus required the presence of functional cellular DNA during their replication. Reich also reported that Rous sarcoma virus (a lipid enveloped-RNA virus) was sensitive to the drug only during the first 10 hours of replication. Barry (11) and White and Cheyne (104) found that fowl plague and influenza virus were sensitive to the drug only during the first 2.5 hours of replication. Pons (75) correlated this sensitivity to actinomycin D with the destruction of replicative forms of influenza virus in the presence of the drug. Reports by Schaffer and Gordon (84) and Cooper (20) on the sensitivity of certain strains of enteroviruses to actinomycin D, also described a drug sensitivity that was manifested only during the first 2 hours of replication. Hog

cholera virus grew in the presence of actinomycin D, according to Aynaud (5). There have been no observations reported on the effect of the drug on BVD replication.

Viral Induced Cell Death

The capacity to kill host cells has been a well recognized property of many viruses. In an early but comprehensive review, Walker (100) suggested that diversions of metabolism induced in the infected cell by the virus-directed synthetic processes were the cause of cell death in most systems. In some instances, however, products of virus-directed synthesis seemed to bring about cellular alterations. These included a viral toxin separable from the adenovirus infectious particles and cellular antigens released from poliovirus-infected cells.

In contrast to normal cells, cells that are killed by viruses do not stain with neutral red. Therefore, neutral red has been routinely used to enhance the visibility of virus plaques. Allison and Malucci (2) observed the effect of infection with a variety of viruses on the uptake of neutral red by cell lysosomes. They correlated this uptake with the degree of staining of the cells and the release of various hydrolytic enzymes from lysosomes. Isolates of Newcastle disease virus that produced red plaques also increased the incorporation of neutral red into cell lysosomes with minor release of enzymes. These effects were reversible with time. Viruses that produced

white (clear) plaques irreversibly inhibited the incorporation of neutral red into lysosomes and caused extensive release of enzymes. Viruses that did not produce plaques failed to affect the lysosomes.

The alteration of lysosomes by mengovirus has been studied by Amako and Dales (4). They observed that a "late" viral protein synthesized between 3 and 5 hours after infection was responsible for the lethal effect on the host cell. The lethal effect was measured by vital staining and was correlated to the leakage of enzymes from lysosomes. Bubel (13) detected cell injury by the leakage of proteins from cells infected with poliovirus. He also reported that a "late" protein produced by the virus from 3 to 5 hours after infection was responsible. Preliminary isolation of this viral protein from the cytoplasm of infected cells has been reported (97). By sequential chemical inactivation of the genome of fowl plague virus, Scholtissek et al. (83) destroyed the ability of this virus to kill cells and to produce neuraminidase. This treatment did not affect the production of viral RNA or nucleocapsid antigen, indicating that the size of the gene for the cytotoxic viral protein was similar to that for neuraminidase.

Bablanian et al. (7) and Amako and Dales (4) have suggested that the capsid protein of poliovirus and mengovirus was cytotoxic. However, infection of certain host cell types with mengovirus resulted in death of the host cells without

production of viral capsid protein (14). Gauntt and Lockart (41) observed that mengovirus killed interferon treated cells even though only a small proportion of the cells were actually synthesizing viral coat protein. They concluded that either the coat protein was not cytotoxic or that it was toxic in such minute amounts as to not be detectable by fluorescent antibody and serum-blocking techniques.

Recent studies using improved techniques to detect macromolecular synthesis have provided additional information about the phenomena associated with virus-induced cell death. Experimental infections with poliovirus (81), Newcastle disease virus (102), herpesvirus (93) and vaccinia virus (72) produced marked inhibitions in cellular DNA, RNA and protein metabolism.

The mechanisms by which alterations in macromolecular synthesis take place has been described for some virus-host cell systems. Mengovirus, an agent very similar to poliovirus, synthesizes an "early" protein which has the properties of a histone (9) and inhibits the cellular transcriptase (38). This results in inhibition of cell-directed RNA synthesis and indirectly produces inhibition of cell protein and DNA synthesis. Adenoviruses synthesize "late" proteins with inhibitory properties (63). The most important of these is the fiber antigen, a protein that is incorporated into the mature virus particle. The fiber antigen binds to the cell DNA and prevents synthesis

of cellular DNA and RNA, thereby indirectly inhibiting protein synthesis.

Attempts to establish an association between virus-induced lysosomal alterations and inhibitions of cellular metabolism have led to the conclusion that even marked inhibitions of cellular DNA, RNA and protein synthesis will produce only delayed cytopathology. Bablanian et al. (6) observed that treatment of poliovirus-infected cells with guanidine did not prevent inhibitions of cellular metabolism but markedly delayed the appearance of cytopathology. According to Plagemann and Swim (74), mengovirus infection of certain cell lines resulted in cell death even though the virus was unable to inhibit the cell transcriptase.

The relationship between virulence in animals and lethal effect in cell cultures has been studied with two strains of mengovirus by Amako and Dales (4). These workers observed that the strain which formed large plaques also produced large amounts of cytotoxic protein and killed mice at approximately 3 days after inoculation. A small plaque strain of virus produced less cytotoxic protein and required a longer time interval (5 to 6 days) to kill mice.

MATERIALS AND METHODS

Cell Cultures

The water used in all procedures and solutions was steam condensed, demineralized, distilled over glass and stored in plastic carboys. It routinely contained less than 0.05 P.P.M. of sodium chloride equivalents when measured by conductivity. All glassware was washed, rinsed and dry-heat sterilized to standards appropriate for tissue culture.

Calf testicles were obtained from a commercial abbatoir in Dubuque, Iowa. The parenchyma of these organs was removed, minced and trypsinized. Dispersed cells were suspended at a concentration of 1.5 million cells/ml in a growth medium that consisted of Hanks' Balanced Salts Solution (HBSS)¹ supplemented with 10% heat inactivated lamb serum¹, 1 g/liter of lactalbumin hydrolysate², 1 X Basal Medium Eagles Vitamin Solution¹ and antibiotics.³ After seeding in 15 × 60 mm tissue culture plastic petri dishes, plastic flasks⁴, or glass tubes, uniform monolayers

¹Grand Island Biological Company, Grand Island, New York.

²Difco Laboratories, Inc., Detroit, Michigan.

³E. R. Squibb and Sons, New York, New York. Antibiotics were added in a concentration of 100 units/ml of penicillin and 100 µg/ml of streptomycin to all solutions requiring bacteriostatic activity.

⁴Falcon Plastics, Los Angeles, California.

with characteristic fibroblastic morphology were obtained in 3 days. Tube cultures were incubated in stationary racks for only 12 to 24 hours to obtain suitable numbers of viable cells for experiments requiring a high multiplicity of infection with virus. All cell cultures were washed once prior to use in any experiment. The washing solution was saline G¹ modified to contain 1 g/liter of lactalbumin hydrolysate and antibiotics. Cell cultures were maintained in a medium consisting of Basal Medium Eagles with glutamine¹ supplemented with 5% heat inactivated horse serum¹, 1.1 g/liter of sodium bicarbonate and antibiotics. This standard maintenance medium was supplemented or substituted by other solutions as needed for individual experiments.

Virus Strains

The Oregon C24V strain (C24V)², and the New York 1 strain (NY1)³ of BVD virus were used in this study. The C24V strain was a cytopathogenic virus used in commercial modified live-virus vaccines while the NY1 strain was a noncytopathogenic virus. The ISU-2 strain of infectious bovine rhinotracheitis

¹Grand Island Biological Co., Grand Island, New York.

²Supplied by Dr. C. J. Welter, Diamond Laboratories, Inc., Des Moines, Iowa.

³Obtained from Dr. J. H. Gillespie, Cornell University, Ithaca, New York.

(IBR-ISU-2), the LCR-4 strain of bovine enterovirus (BE-LCR-4), and vesicular stomatitis virus (VSV) were used as test viruses.¹

The C24V strain was passaged an undetermined number of times in cell cultures of bovine origin while the NY1 strain was passaged twice in bovine kidney cells when received. Both strains were used throughout this study at the third to seventh passage level in primary bovine testicle cells. Lots of each of these viruses were grown in 250 ml plastic bottles and harvested at the time the monolayers exhibited 75% CPE or at 4 days in the case of NY1 virus. Each bottle was frozen and thawed once and the fluids were harvested, centrifuged at low speed to remove cell debris and stored at -70 C. Sham lots were prepared in the same way as the NY1 virus lots except no virus was inoculated.

Infectivity Assays

All virus strains were assayed for infectivity by a plaque method with the exception of the NY1 strain. Two petri dishes with cell monolayers were exposed to 1 ml aliquots of each dilution of virus in saline G. The virus was adsorbed for 1 hour at room temperature and was then discarded. The monolayers were overlaid with 5 ml of maintenance medium containing 1%

¹Stock virus preparations from the Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa.

Noble agar¹. After incubation until visible virus plaques were seen, a second overlay consisting of 0.01% neutral red and 1% Noble agar in water to render the virus plaques highly visible was added.

An interference test was used to assay infectivity with the NY1 strain. One milliliter aliquots of each virus dilution were adsorbed onto 3 replicate plates, and the monolayers were covered with 2 ml of the standard maintenance medium. After incubation for 3 days, the medium was discarded and the plates challenged with a known quantity of the C24V strain. A reduction in the number and size of plaques formed by the challenge strain was taken as evidence of the presence of NY1 virus and the titer was expressed as the 50% infectious dose (ID_{50} /ml).

Characterization of Virus Strains

The C24V and NY1 strains were characterized serologically and biochemically to confirm their classification as BVD strains. Virus neutralization tests, using the constant virus (approximately 100 infectious units) - decreasing serum technique, were performed with an antiserum prepared in rabbits against the NADL strain of BVD virus. The endpoint read was the dilution of serum that completely neutralized the infectivity of the fixed quantity of virus.

¹Difco Laboratories Inc., Detroit, Michigan.

Tests for sensitivity to lipid solvents were conducted by mixing 2 ml of chloroform with 8 ml of a chilled virus dilution and shaking thoroughly for 10 minutes. After low speed centrifugation to separate the chloroform to the bottom phase, the upper 1 ml of the aqueous phase was withdrawn and assayed for infectivity. A 10 ml aliquot of the same virus dilution was shaken, centrifuged, assayed in the same manner, and served as an untreated control. The BE-LCR-4 strain, a known chloroform-resistant virus, was run in parallel to provide a negative control for the test.

To determine the type of nucleic acid present in the viruses, titrations were performed using a plaque or plaque interference method with 0.05 μ M/ml of 5-iododeoxyuridine¹ in the maintenance overlays. Control titrations were run simultaneously without drug treatment. The IBR-ISU-2 strain was used as a known DNA virus that was inhibited by the thymine analogue when tested in parallel.

Assay for Interference

To test for viral interference in various preparations, a procedure similar to the plaque reduction assay for interferon (76) was used. Washed cell monolayers grown in petri dishes were covered with 1 ml aliquots of the material to be tested.

¹Nutritional Biochemicals Corporation, Cleveland, Ohio.

Eight hours later the material was discarded, and the monolayers were challenged with the appropriate virus. The cultures were overlayed with agar-containing maintenance medium as described previously. Activity was measured as percent of average plaque reduction with respect to appropriate controls. In most cases 4 replicate plates were used for each preparation tested.

Separation of Infectivity from Interfering Activity

Several procedures were used in attempts to separate infectious virus particles from the interfering activity demonstrated by the NY1 strain. Filtration through 25 nm pore filters¹ was performed using micro-syringe filter holders¹. Test material was also dialyzed against a pH 2.0 saline solution for 48 hours. It was then dialyzed back to pH 7.2 in HBSS lacking phenol red for 48 hours at 4 C. To irradiate materials with ultraviolet light, a volume of sample that gave a layer of 2 mm in thickness in a petri dish was exposed to $2100 \text{ erg} \times \text{sec}^{-1} \times \text{cm}^{-2}$ for 10 minutes on a rotator at 210 rpm. Ultracentrifugation techniques were also used to remove virus particles from the supernatant fluid of test materials. Samples were centrifuged at 100,000 G for 1 hour in the No. 40 angle rotor or at 120,000 G for 20 hours in the SW56 swinging bucket

¹Millipore Corporation, Bedford, Massachusetts.

rotor of a Beckman L2-65 centrifuge. Column chromatography was also used in these attempts to separate infectivity from interfering activity. Beds of Biogel P30, P60 and P150¹ were prepared in 300 × 25 mm descending chromatography columns in HBSS. Chromatographed samples were either unpurified virus lots or virus lots that had been passed through 25 nm filters. These were concentrated by ultrafiltration on Diaflo UM-3 membranes², which had a cutoff point of 1,000 daltons. Since each sample consisted of horse serum-containing standard maintenance medium plus viral and cellular products, the globulins and albumin of the serum were used as markers of known molecular weight. These were easily detected in the eluted fractions by their absorbance at 280 nm on an ISCO model UA-2 flow analyzer³. The elution buffer used was HBSS, prepared without phenol red to minimize background absorption.

For one experiment, ultrafiltration on XM50 membranes with a cutoff point of 50,000 daltons was utilized to obtain virus samples free of measurable interfering activity of low molecular weight. The unpurified virus was diluted 1:5 in HBSS and concentrated back to its original volume by ultrafiltration. All procedures were carried out in such a way as to minimize

¹Calbiochem., Los Angeles, California.

²Amicon Corporation, Cambridge, Massachusetts.

³Instrumentation Specialties Company, Lincoln, Nebraska.

bacterial growth in the fractions. All fractions were sterilized by filtration before use in interference assays.

Virus Purification

For experiments where high multiplicities of infection (m.o.i) of C24V or NY1 virus were required, the virus-containing maintenance medium was clarified by centrifugation at 10,000 G for 10 minutes to remove subcellular particles. No substantial loss of infectivity resulted from this treatment. The supernatant material was further purified by gel filtration on Biogel P300 chromatography columns using HBSS as the elution buffer. Infectivity was excluded from these columns as a fraction that could be detected by its light-scattering properties and which was separable from the serum protein-containing fractions detected by their absorbance at 280 nm. This procedure routinely yielded 100% of the initial infectivity as partially purified virus that was freed of material of 300,000 daltons or less. It was stored at -70 C until used.

Virus Growth Curves

Cell sheets grown for 48 hours in 30 ml flasks were used for virus growth curve experiments. Ten replicate flasks were infected with 2 ml of maintenance medium containing a 1/20 to 1/50 dilution of C24V or NY1 virus providing a m.o.i. of 0.4 infectious units per cell. The flasks were returned immediately

to the 37 C incubator and the time recorded as 0 hours. At 2 hours the medium was discarded and all flasks were washed 5 times with 1 ml aliquots of warm saline G. This step removed all unadsorbed virus particles and was followed by the addition of 2 ml of warm maintenance medium. Each of two flasks were sampled for intracellular and extracellular virus every 3 hours, from 3 to 15 hours after infection. To sample for extracellular virus, the 2 ml of medium from each flask were collected in sterile vials. Two ml of maintenance medium were added to the infected cell sheets and the flasks were frozen in an ethanol-dry ice mixture. Rapid thawing in a 37 C waterbath disrupted the cells and released intracellular virus into the medium. The samples for extracellular virus were also submitted simultaneously to a freezing and thawing cycle to account for any loss in infectivity due to this process. The samples were frozen again in ethanol-dry ice and stored at -70 C until they were assayed. Infectivity assays were performed on each sample as described previously and the results recorded as the log of PFU/ml or ID₅₀/ml. An additional growth curve experiment was performed with partially purified C24V by the procedure delineated above. In this experiment the m.o.i. was 4.0 and 5 samples were taken beginning at 7 hours after infection and at 8 hour intervals thereafter.

Effect of Actinomycin D on C24V Virus Growth

A modification of the procedure used for virus growth curve studies was applied to determine the effect of actinomycin D¹ on the growth of C24V virus. Replicate flasks that were overlaid with standard maintenance medium 1 hour prior to infection, 2 were treated with 0.1 µg/ml of actinomycin D for the duration of the experiment, 2 were treated with the same level of drug from 3 hours after infection onward and 2 were left untreated. All flasks were infected simultaneously with a m.o.i. of 0.03 and processed by methods utilized in virus growth curve experiments. A sample of extracellular virus was taken from each flask at 3 hours, fresh maintenance medium was added, and another sample of extracellular virus was taken at 12 hours.

Leighton tubes containing 12-hour cell cultures were infected with a m.o.i. of 20 of partially purified C24V or NY1 virus in 1 ml of standard maintenance medium or were left uninfected in the same medium. At specified times after infection, tubes were pulsed with 0.2 ml of saline containing 3 µc of uridine-5-H³ (24c/mM)² in an 800-fold excess of unlabeled thymidine to prevent the incorporation of label into

¹Merck and Company, Incorporated, Rahway, New Jersey.

²New England Nuclear Corporation, Boston, Massachusetts.

DNA. Exactly 30 minutes after pulsing, the coverslips were fixed in ice-cold Carnoy's fixative for 10 minutes. The coverslips were transferred to 70% ethanol and held at 4 C until processed further. Some coverslips were processed without a pulse to obtain data for background corrections. All coverslips were treated in 5% trichloroacetic acid for 10 minutes at 4 C, rinsed twice in distilled water, air dried, and mounted cell-side up on microscope slides with Permount¹. Autoradiographs were prepared by dipping each slide in melted NTB-3 liquid emulsion² diluted in an equal volume of water and exposing the films for 3 days at -18 C in a light-tight box (1). Slides were developed, fixed and stained through the emulsion film with Giemsa stain.

All slides were examined under the microscope at a magnification of 2,000 and the number of grains contained in the cytoplasm and nucleus of each of 100 randomly chosen cells was recorded. The number of grains counted in 10 random microscope fields containing no cells was also recorded for each slide. Each nuclear and cytoplasmic count was corrected for background radiation by using average background values proportional to the number of grains per field found in that slide. Corrections for cell compartment geometry and self

¹Fisher Scientific Company, Fair Lawn, New Jersey.

²Eastman Kodak Company, Rochester, New York.

absorption were performed on each count as described by Perry et al. (73). Adjusted counts were used for appropriate statistical analysis.

Metabolic Inhibitions

Disposable glass tubes containing 24-hour cell cultures were used to measure the rates of synthesis of DNA, RNA and protein in C24V or NY1 virus-infected cells. The tubes were infected with a m.o.i. of 2.2 of partially purified C24V or NY1 virus in 1 ml of standard maintenance medium or were left uninfected in the same medium. Radiotracer pulses in 0.2 ml of saline G were added at specified times. DNA synthesis was measured with 4 μ c pulses of thymidine-methyl-5- H^3 (67.5c/mM)¹, RNA synthesis with 3 μ c pulses of uridine -5- H^3 (24c/nM)¹ in an 800-fold excess of unlabeled thymidine, and protein synthesis was measured with 1 μ c pulses of a mixed UL-C¹⁴-amino acid mixture². Following a 30 minute pulse the tubes were removed from the incubator, frozen in liquid N₂ and stored at -18 C. Unpulsed tubes were also treated in this manner and used for background determinations. The tubes were thawed in ice-cold water and 1 ml of 20% ice-cold trichloroacetic acid was added with thorough mixing. After the tubes were allowed to stand

¹New England Nuclear Corporation, Boston, Massachusetts.

²International Chemical and Nuclear, City of Industry, California.

for 10 minutes at 0 C, they were centrifuged for 5 minutes at 200 G. The precipitate was washed in cold 80% ethanol followed by absolute ethanol with 50% ether. The washed precipitates were solubilized in 1 ml of NCS solution¹, 14 ml of toluene-based scintillation solution was added and the mixture transferred to glass counting vials. Activity was measured as counts per minute (CPM) in a TriCarb 3375 liquid scintillation spectrometer². These were corrected for quenching effects to disintegrations per minute (DPM) with correction factors derived from calibration curves. The DNA, RNA and protein synthesis rates in infected cells were expressed as per cent of control synthesis rates.

To test for specificity of labeling, frozen tubes were thawed in a 37 C waterbath and 1 ml of a 0.1% solution of deoxyribonuclease³, a 0.01% solution of pancreatic ribonuclease³ or 0.2% trypsin in saline were added. Digestion was allowed to proceed for 30 minutes at 37 C and was terminated by chilling and precipitation with trichloroacetic acid. Processing for counting of radioactivity was by procedures indicated previously.

¹Amersham-Searle, Des Plaines, Illinois.

²Packard Instrument Company, Downers Grove, Illinois.

³Nutritional Biochemicals Corporation, Cleveland, Ohio.

Leakage of Proteins

To measure the leakage of proteins from virus-infected cells, 18 hour cell cultures in 60 × 15 mm plastic petri dishes were labeled with 1 μ c of UL-C¹⁴-leucine (180 mc/mM)¹ in 2 ml of HBSS containing 5% inactivated horse serum.² Following exposure of the cells to the label for 18 hours, the medium was discarded and the plates washed 3 times with saline G to eliminate unbound label.

Cultures were then infected with a m.o.i. of 1.3 of partially purified C24V or NY1 virus in 2 ml of standard maintenance medium or left uninfected as controls. At specified times, the supernatant from these plates was harvested to determine the amount of leakage of labeled cellular proteins. The supernatant fractions were centrifuged at 200 G for 5 minutes to eliminate free cells and were frozen in liquid N₂ prior to storage at -18 C. The fractions were thawed and the acid-insoluble label was precipitated with 2 ml of ice-cold 20% trichloroacetic acid. These precipitates were further processed for scintillation counting as previously described, counted, and the activity expressed as DPM of extracellular C¹⁴-labeled protein.

¹Schwartz Bioresearch, Inc., Orangeburg, New York.

²Grand Island Biological Company, Grand Island, New York.

Inhibition of Protein Leakage

Puromycin¹, a translation inhibitor, and actinomycin D, a transcription inhibitor, were used in an attempt to inhibit the leakage of cellular proteins induced by C24V virus infection. Cultures of C¹⁴-protein labeled cells were infected with a m.o.i. of 3.5 of C24V virus in 2 ml of standard maintenance medium. Pulses of 50 µg/ml of puromycin or 0.1 µg/ml of actinomycin D in 2 ml of warm maintenance medium were added to plates at specified times, after removing the 2 ml of virus inoculum. Sham pulses of maintenance medium without drug were added to another set of cultures. The duration of the pulses was 1 or 2 hours and at the end of this period the pulsed plates were washed twice with warm saline G. The cultures were overlayed with 2 ml of warm maintenance medium and incubated until 30 hours following infection. At that time all supernatants were harvested for protein leakage determinations as described previously.

¹Nutritional Biochemicals Corporation, Cleveland, Ohio.

RESULTS

Characterization of Virus Strains

The C24V and NY1 strains were neutralized by a 1:32 dilution of the rabbit anti-BVD-NADL serum. As shown in Table 1, both viruses were sensitive to chloroform but were not affected by the addition of iododeoxyuridine. This confirmed that both were RNA viruses that possessed lipid envelopes and antigenic characteristics of BVD viruses.

Table 1. Characterization of C24V and NY1 viruses by chloroform sensitivity and growth in the presence of iododeoxyuridine

Virus Strain ^a	Untreated	Chloroform 20%	Iododeoxyuridine 0.05 μ M/ml
C24V ^b	1.1×10^4	0	3.5×10^3
NY1 ^c	1.1×10^7	5.6×10^0	3.2×10^6
BE-LCR-4 ^b	8.0×10^5	5.0×10^5	N.D. ^d
IBR-ISU-2 ^b	1.2×10^5	N.D. ^d	0

^aC24V and NY1 strains of BVD virus, LCR-4 strain of bovine enterovirus and ISU-2 strain of infectious bovine rhinotracheitis.

^bTiter in PFU/ml.

^cTiter in ID₅₀/ml.

^dNot done.

Filtration experiments were conducted to determine the ability of BVD virus infectivity to pass a 25 nm filter. A reduced but detectable amount of infectivity from both viruses passed the filter (Table 2).

Table 2. Filtration of C24V and NY1 viruses through 25 nm filters

Virus	Unfiltered	Filtered
C24V	1.3×10^4 ^a	4×10^1
NY1	1.8×10^7 ^b	7×10^2

^aTiter in PFU/ml.

^bTiter in ID₅₀/ml.

Unfiltered and 25 nm filtered C24V virus was characterized by dialysis to pH 2, trypsin digestion and ribonuclease digestion. In Table 3, the results of such tests are presented. The 25 nm filtrable infectivity resembled unfiltered virus in its sensitivity to pH 2 and resistance to ribonuclease attack, but was more sensitive to trypsin activity.

Table 3. Comparison of characteristics of unfiltered and 25 nm filtered C24V virus

Virus	No treatment	Dialysis to pH 2	Trypsin ^a Digestion	Ribonuclease ^b Digestion
Unfiltered C24V	4.3×10^4	0	1×10^4	5.1×10^4
25 nm filtered C24V	2.8×10^2	0	0	2.8×10^2

^a0.1% trypsin for 30 minutes at 37 C, stopped by adding 2 mg/ml of soybean trypsin inhibitor and chilling.

^bRibonuclease 50 µg/ml for 30 minutes at 37 C.

Separation of Infectivity from Interfering Activity

Since filtration through 25 nm filters did not eliminate infectivity, other procedures were required. Gel filtration was resorted to in an attempt to prepare fractions which had interfering activity but were free of infectivity. Typical elution profiles from P60 columns are depicted in Figure 1. Unexpectedly, the 25 nm filtered sham material possessed low levels of activity in 2 peaks, one of them excluded and the other retained by the column. No enhancement of challenge virus was seen with any fractions from sham materials. Fractions obtained from the ascending part of a NY1 curve gave enhancing activity. However, fractions from the peak and descending part of the curve yielded materials with interference activity.

Figure 1. Interfering activity against C24V virus in fractions obtained from P60 columns. Samples of NY1 virus, C24V virus or sham material were filtered through 25 nm membranes and concentrated 14 X by ultrafiltration. The interfering activity of each fraction was recorded as percent plaque reduction with respect to control petri plates. Enhancement was recorded as \log_{10} of fraction from controls. The horse serum proteins were detected by absorbance at 280 nm

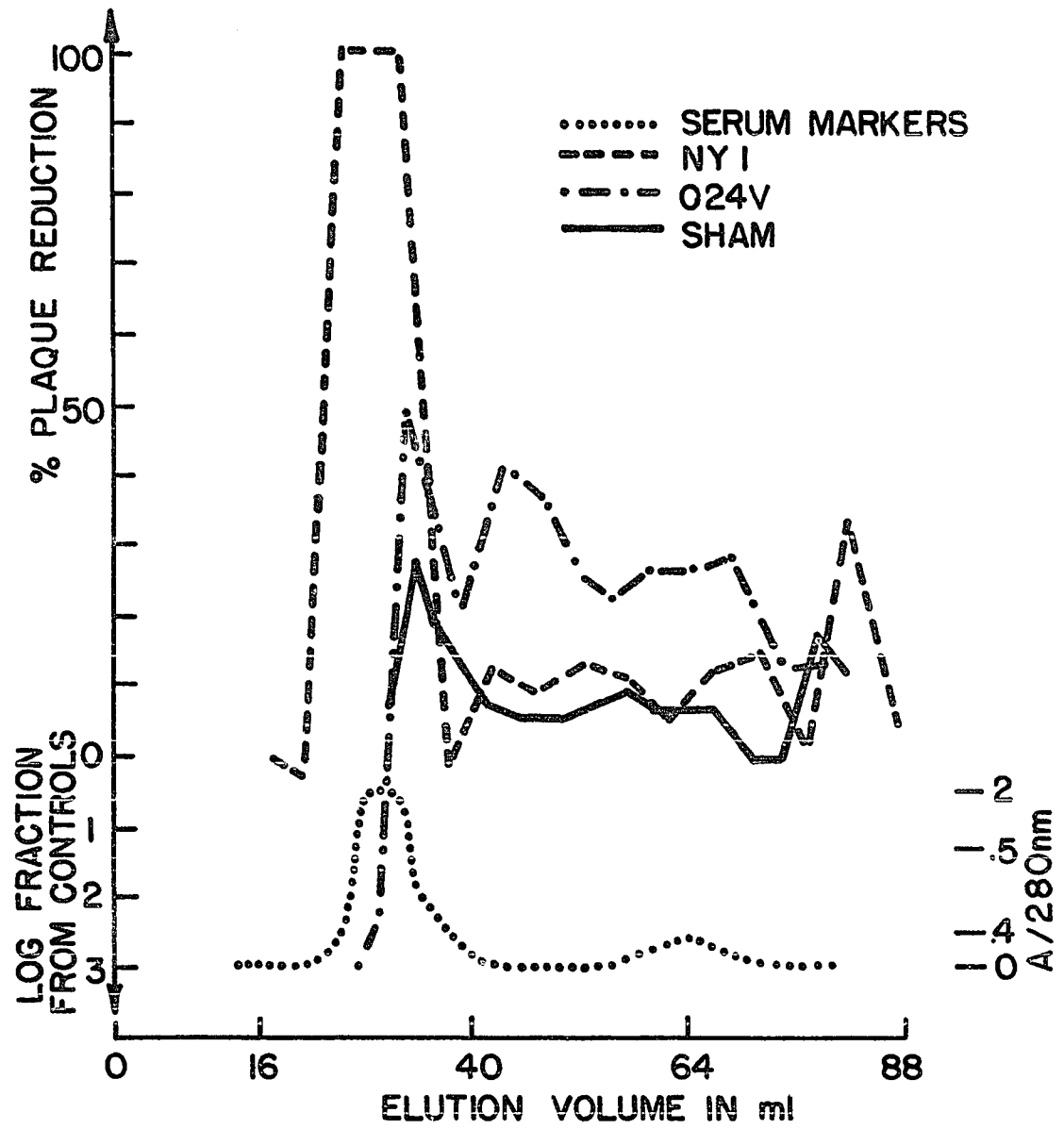
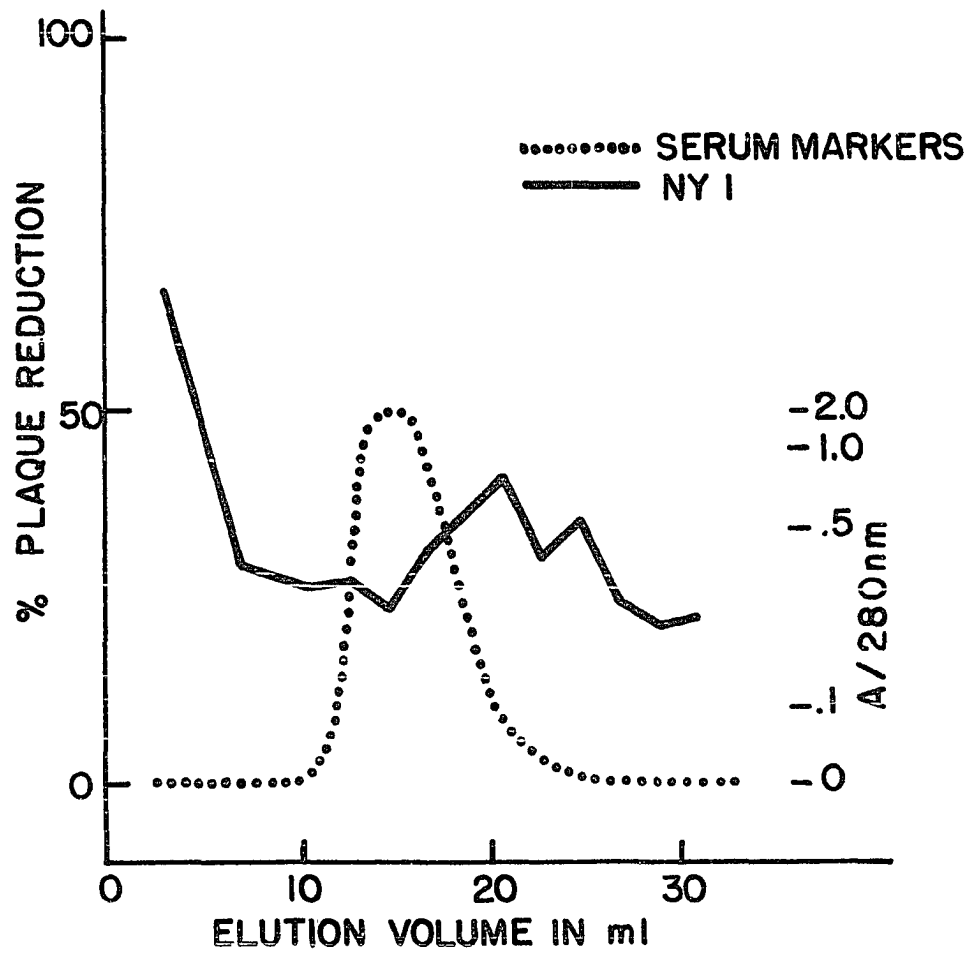


Figure 2. Interfering activity against C24V virus
in fractions obtained from a P30 column.
The sample used was NY1 virus filtered
through 25 nm membranes



Although assays for infectivity were not performed, it was assumed that some infectious virus was present. This assumption was made since viral infectivity passed through 25 nm filters and was excluded from the P60 column. Assays of fractions from the retained peak also showed interfering activity. It was assumed that these fractions were free of infectious virus since the corresponding C24V fractions lacked infectivity. A number of peaks of interfering activity were obtained from a 25 nm filtered C24V sample. A fraction that had a strong enhancing effect on the C24V challenge was collected from the column in advance of those fractions with interfering activity. This enhancing effect was due to the presence of 25 nm filtrable infectivity in the sample.

A 25 nm filtered NY1 virus preparation passed through a P30 column yielded a fraction that eluted following all detectable horse serum protein markers (Figure 2). Retention of this material on the column would indicate a molecular weight of 30,000 or less.

Centrifugation and filtration were utilized to further characterize the interfering activity of pH 2 dialyzed NY1 and C24V materials. Preparations that were filtered or centrifuged after pH 2 dialysis were assayed for interfering activity and compared to controls. A Poisson distribution of plaque counts was assumed and a Chi-square test (89) was used to determine the significance of the observed differences. Significant

activity was seen in all pH 2 dialyzed materials as evidenced in Table 4. No differences in activity were noticed after filtration through a 25 nm filter or ultracentrifugation at 100,000 G for 1 hour.

Table 4. Interfering activity against C24V virus of pH 2 dialyzed NY1 virus before and after removal of viral components

Treatment	Average plaque counts	Percent plaque reduction
Maintenance medium	82.7	
pH 2 dialyzed virus	25.5 ^a	69.2
pH 2 dialyzed - 25 nm filtered virus	29.7 ^a	64.2
pH 2 dialyzed - centrifuged virus (100,000 G for 1 hour)	34.0 ^a	58.9

^aStatistical comparisons of plaque counts between pH 2 dialysed NY1 virus and maintenance medium ($p < 0.005$).

The interfering activity of pH 2 dialyzed NY1 preparations against various viruses is shown in Table 5. A significant reduction in the number of challenge plaques of C24V and VSV in comparison to maintenance medium-treated plates was noted, but the material had no significant effect on IBR or BE viruses.

Table 5. Antiviral spectrum of the interfering activity contained in pH 2 dialyzed NY1 preparations

Challenge virus ^a	Average plaque counts		
	Maintenance medium	pH 2 dialyzed NY1 preparations	Percent plaque reduction
C24V	19.5	7.0 ^b	64.0
VSV	25.3	6.7 ^b	73.5
IBR-ISU-2	89.3	79.0	11.5
BE-LCR-4	95.0	93.0	2.1

^aC24V strain of BVD, vesicular stomatitis, ISU-2 strain of infectious bovine rhinotracheitis and LCR-4 strain of bovine enterovirus.

^bStatistical comparisons of plaque counts between pH 2 dialyzed NY1 preparations and corresponding maintenance medium ($p < 0.005$).

A number of sham lots obtained from different batches of cells were also examined for interfering activity that was resistant to pH 2 dialysis and active against VSV. Low levels of activity were present in all lots prepared before bacterial growth was detected on the walls of the plastic carboys used to store water for tissue culture use. After the plastic carboys were replaced by sterile glass containers to restrict the growth of bacteria, the sham and virus lots prepared did not contain interfering activity that was pH 2 resistant, active against VSV and of small molecular weight.

Association of Interference with Infectivity

In some assays cultures infected for 3 days with NY1 virus were collected and tested for infectivity. In every case and in all NY1 dilutions tested, those fluids from cultures that resisted the challenge dose of C24V also contained infectious virus. Fluids from non-interfering cultures did not contain infectious NY1 virus. When interfering monolayers were washed 5 times prior to challenge, they remained refractory to the challenge virus.

Ultracentrifugation and irradiation with ultraviolet light were used in attempts to remove the interfering activity associated with large molecular weight components of NY1 virus preparations. These preparations were first treated by ultrafiltration to remove materials with interfering activity that had a molecular weight of less than 50,000. The dose of ultraviolet irradiation used was sufficient to reduce the infectivity of a parallel sample of C24V virus from 8×10^6 to 0.5 PFU/ml and ultracentrifugation reduced the infectivity of another sample from 8×10^6 to 1.5×10^2 PFU/ml. The m.o.i. of NY1 virus used in these trials was 0.2 which was sufficient to infect 12% of the cells in each petri plate with one infectious particle. These results are presented in Table 6.

Significant interfering activity was produced by purified virus and irradiated purified virus preparations with only a slight difference detected between their activities.

Ultracentrifugation removed all interfering activity from the supernatant fluid. The interfering activity of these preparations was associated with either infectious or irradiated viral particles.

Table 6. Interfering activity against C24V virus in ultracentrifuged or ultraviolet irradiated XM50-purified NY1 virus

Treatment	Average plaque counts	Percent plaque reduction
Maintenance medium	129.7	
NY1 virus	39.7 ^a	69.4
NY1 virus, supernate of ultracentrifugation (120,000 G, 20 hours)	139.7	<0
NY1 virus, irradiated	62.0 ^a	52.2

^aStatistical comparisons of plaque counts between NY1 materials and maintenance medium ($p < 0.002$).

The interfering activity of large molecular weight components in NY1 and sham preparations was determined by the effect on C24V and VSV viruses. These preparations were obtained by filtration through 25 nm filters and subsequent exclusion from P150 columns to remove materials of molecular weight smaller than 150,000. Results are presented in Table 7.

Table 7. Interfering activity against C24V and VSV viruses in NY1 and sham preparations containing only large molecular weight components

Treatment	Average plaque counts	
	C24V challenge	VSV challenge
HBSS buffer	94.3	51.0
NY1 virus	51.7 ^a	34.0
Sham lot	64.7	35.6

^aStatistical comparisons of plaque counts between NY1 virus and the corresponding sham ($p < 0.05$).

The NY1 and sham preparations showed significant interfering activity against both viruses. However, a fraction from a sham lot excluded by a P60 column (Figure 1) showed a corresponding peak of activity. Low levels of activity were detected against C24V virus but not against VSV virus in the NY1 virus preparation. This was evidence that NY1 virus preparations contained large molecular weight components which interfered with the homologous C24 V strain but did not interfere with other viruses.

Virus Growth Curves

The kinetics of viral replication were very similar with NY1 and C24V viruses. After an intracellular phase of 10 to 11

hours, virus was released from cells as shown in Figure 3. Progeny intracellular and extracellular virus appeared at the same time, indicating that there was no intracellular accumulation of infectious virus.

The results of experimentation establishing a growth curve at higher m.o.i. are shown in Figure 4. Accumulation of intracellular virus was not detected in this case either and the time of release of virus was comparable to that in the previous experiments. Also indicated in this figure are the times at which various cytopathologic changes were observed in cells infected with virus at a m.o.i. = 10. When unpurified C24V virus was used, a slight but transient cell shrinkage was observed 1 hour after infection. This effect was not observed when purified virus was used. Cellular alterations were observed at 19 hours after infection and cells detached after 30 hours. No cytopathology of either transient or permanent nature was seen with any multiplicity of NY1 virus.

The effect of increased m.o.i. on the yield of extracellular virus after a single cycle of replication was determined in cell cultures infected with C24V virus at a m.o.i. of 1.3 or 90. The yield of virus from duplicate plates at 12 hours is presented in Table 8. No viral interference was demonstrated with this high m.o.i.

Figure 3. Growth curves of NY1 and C24V virus. Cells were infected with a m.o.i = 0.4. Samples from duplicate flasks are averaged for each point

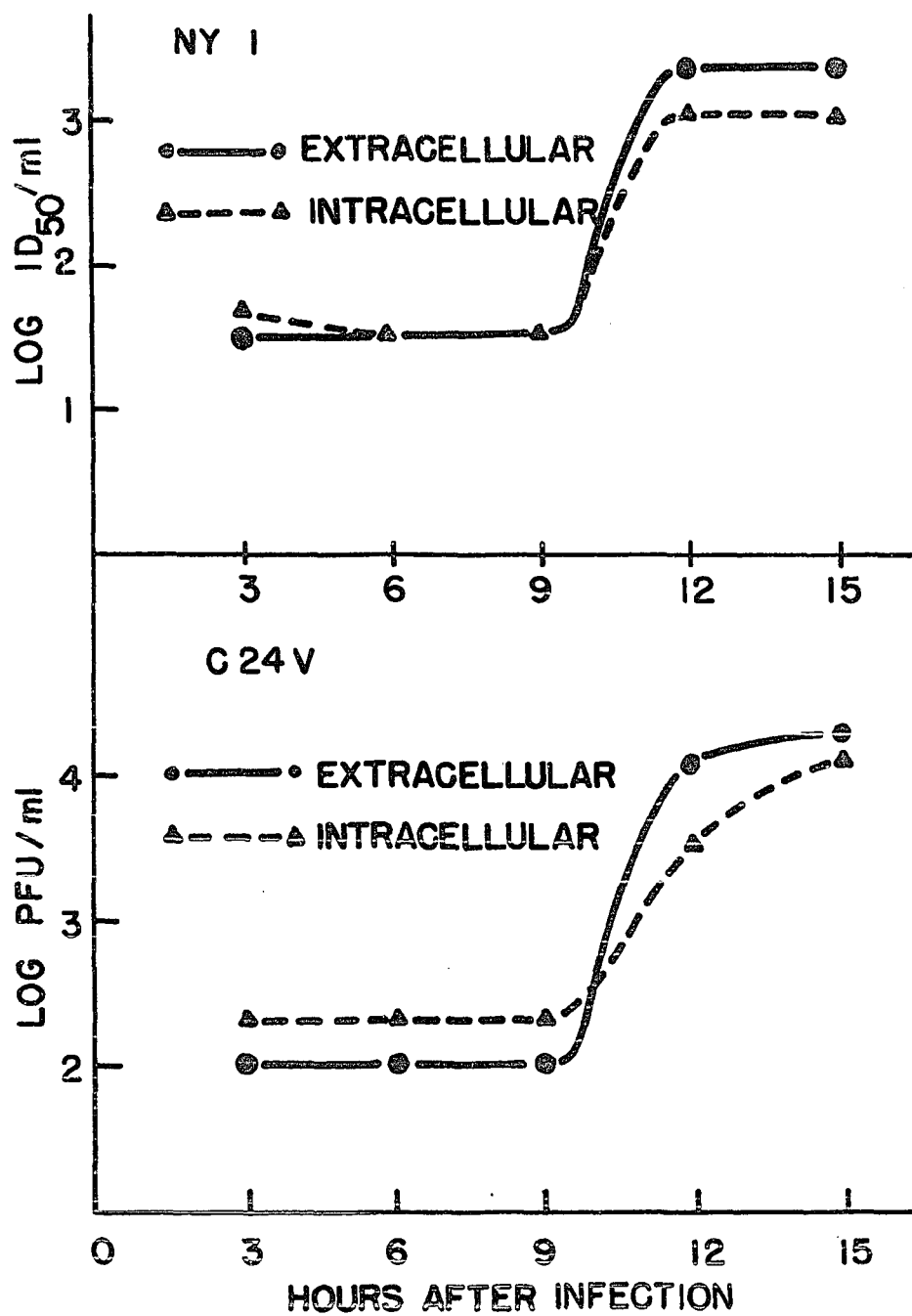


Figure 4. Growth curve of C24V virus. Cells were infected with a m.o.i. = 4 of partially purified (P300 excluded) virus. Samples from duplicate flasks are averaged for each point. Observations for cytopathology were performed under phase optics on cells infected with a m.o.i. = 10 of unpurified or partially purified C24V virus

C24V VIRUS GROWTH CURVE

—•— EXTRACELLULAR
- - - INTRACELLULAR

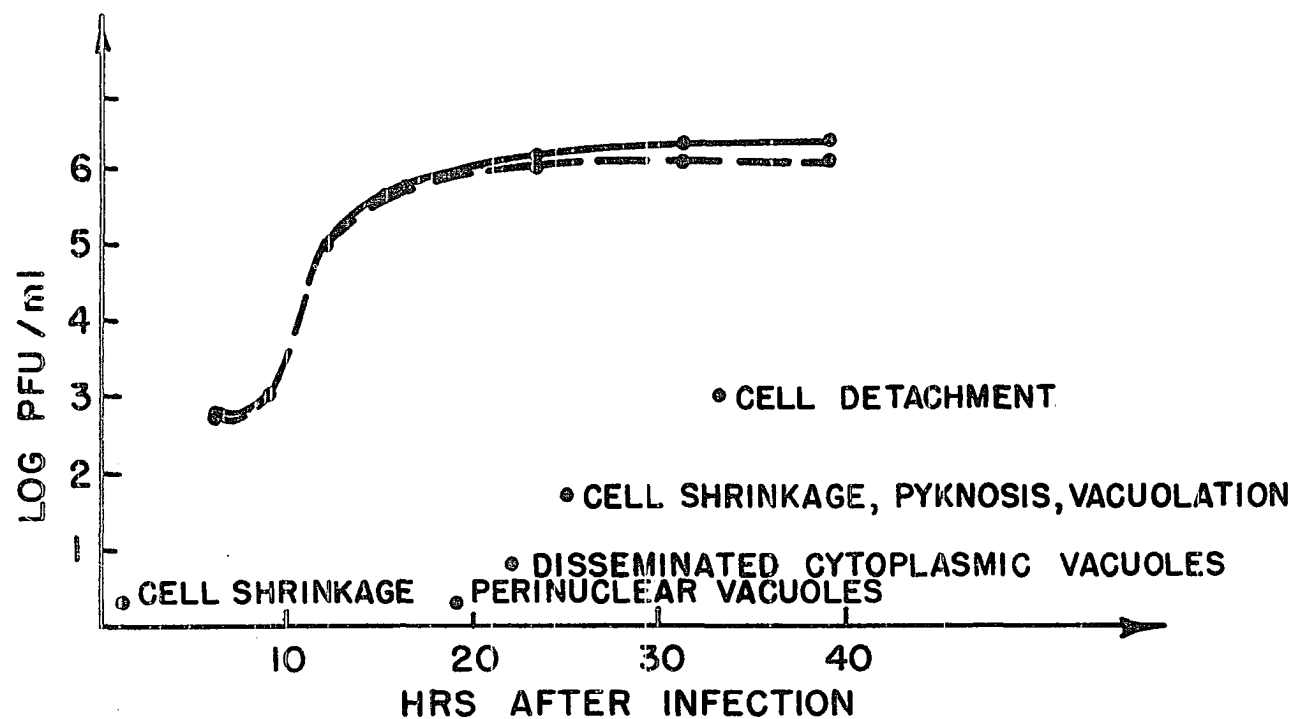


Table 8. Yield of extracellular C24V virus from cells infected with high and low m.o.i. after a single cycle of replication

Multiplicity of Infection	Yield in PFU/ml
1.3	6×10^5
90	1.2×10^6

Growth of Virus in the Presence of Actinomycin D

Presence of actinomycin D in the medium during the entire growth cycle of C24V caused a distinct decrease in the yield of virus. However, addition after the first 3 hours of infection did not influence the yield of virus as compared to untreated controls (Table 9).

Table 9. Yield of extracellular virus from duplicate C24V infected cell cultures treated with actinomycin D

Experiment	Actinomycin D	Average PFU/ml	
		3 hours	12 hours
1	No treatment	1.7×10^2	1.1×10^5
	From -1 to 12 hours	2.3×10^2	5.6×10^3
	From 3 to 12 hours	2.0×10^2	1.0×10^5
2	No treatment	1.1×10^2	1.0×10^4
	From -1 to 12 hours	1.2×10^2	1.5×10^3
	From 3 to 12 hours	1.0×10^2	1.0×10^4

Site of RNA Synthesis

The rate of RNA synthesis in the nucleus or cytoplasm of infected or control cells was measured. In Table 10, the average number of autoradiograph grains in each cell compartment was recorded in duplicate cultures infected for 14 hours with a multiplicity of 20 for each virus. No significant differences were detected between cytoplasmic counts. Nuclear counts of cells infected with either virus were significantly increased over those of uninfected cells.

Table 10. Mean RNA grain counts per cell compartment in cells infected for 14 hours and then pulsed for 30 minutes with uridine-5-H³

Cell Compartment	Virus Used		
	C24V	NY1	Uninfected
Cytoplasm	1.80	2.73	2.38
Nucleus	14.80 ^a	12.74 ^a	6.82

^ap<0.05.

Alterations in Metabolism of Infected Cells

The specificity of radiolabeling of DNA, RNA and protein was determined by experimentation utilizing enzymatic digestion. Results of such tests on 30 minute pulse-labeled cells are

summarized in Table 11. Counts of radioactivity incorporated into acid-precipitable compounds were reduced by specific digestion with the appropriate enzymes. Excess thymidine addition to uridine- H^3 label was effective in preventing non-specific incorporation into DNA. The trypsin preparation was observed to contain apparent RNase activity.

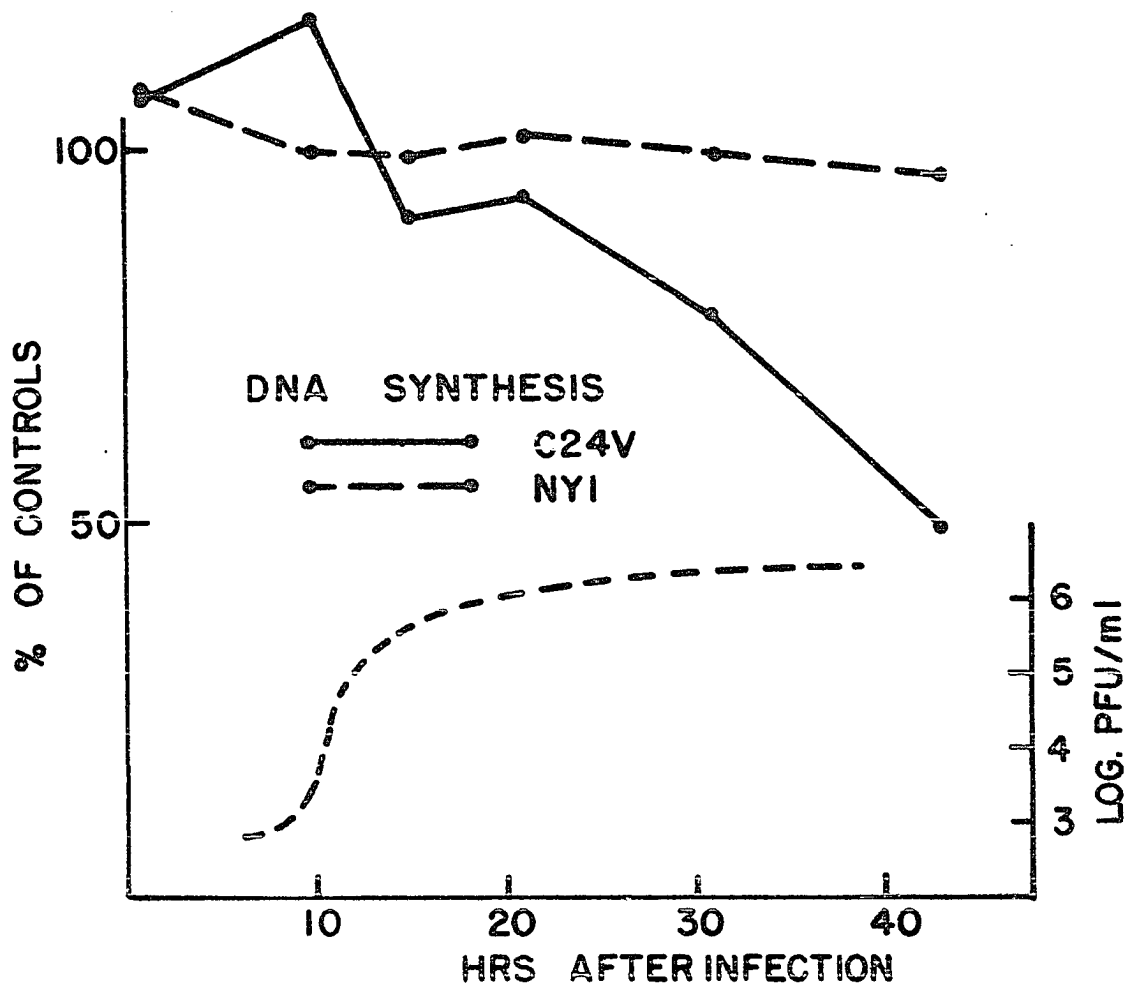
Table 11. Specificity of thymidine- H^3 , uridine- H^3 and C^{14} -amino acid mixture labeling of cells measured as remaining activity in percent after enzymatic digestion

Label used	Enzyme		
	DNase	RNase	Trypsin
Thymidine- H^3	12	84	N.D. ^a
Uridine- H^3 with 800 X excess thymidine	83	12	10
C^{14} -amino acid mixture	N.D. ^a	86	42

^aNot done.

The influence of viral infection on the synthesis of host cell DNA can be seen in Figure 5. The NY1 strain did not alter the synthesis of cellular DNA at any time during the observation period of 43 hours. In contrast, the cytopathogenic C24V strain significantly inhibited host cell DNA synthesis. This inhibition was observed long after initial release of virus

Figure 5. Cellular DNA synthesis in cultures infected with C24V or NY1 virus. Both viruses were added to cultures at a m.o.i. = 2.2. Triplicate samples were measured at the designated times. A growth curve for C24V virus is plotted as a reference. Significant differences ($p < 0.025$) were detected between both virus-infected cultures only at 43 hours after infection



from cells since it was detected only in 43 hour samples. Total RNA synthesis was measured in cell cultures without discriminating as to whether it was cell or virus directed synthesis. No difference was detected between uninfected and NY1 infected cultures (Figure 6). Cultures infected with C24V virus had decreasing rates of synthesis of total RNA but the change was not significantly different during the period of observation. Total protein synthesis levels are recorded in Figure 7, as the sum of cell and virus-directed synthesis. Protein synthesis in infected cells did not differ from that of uninfected cultures.

Virus-induced Protein Leakage

Uninfected cultures of primary bovine testicle cells continuously released proteins which accumulated in the medium (Figure 8). Cultures infected with NY1 exhibited a similar leakage while those infected with C24V released larger amounts of cellular protein. These differences were significant at 30 and 37 hours after virus infection.

The amount of cell protein leaked was influenced by pulses of puromycin or actinomycin D (Figure 9). Cell protein leakage was determined at 30 hours irrespective of the time that pulses were given. Pulses of puromycin within 4 hours of infection did not alter the amount of leakage of cellular proteins, but

later pulses significantly decreased leakage. Pulses of actinomycin D increased the amount of protein leakage. As the time of addition of actinomycin D increased, the amount of protein leakage also increased.

Figure 6. Total RNA synthesis in cultures infected with C24V or NY1 virus. The m.o.i. was 2.2 for both viruses. Triplicate samples were measured at the designated times. A growth curve for C24V virus is plotted as a reference. No significant differences were detected between viruses

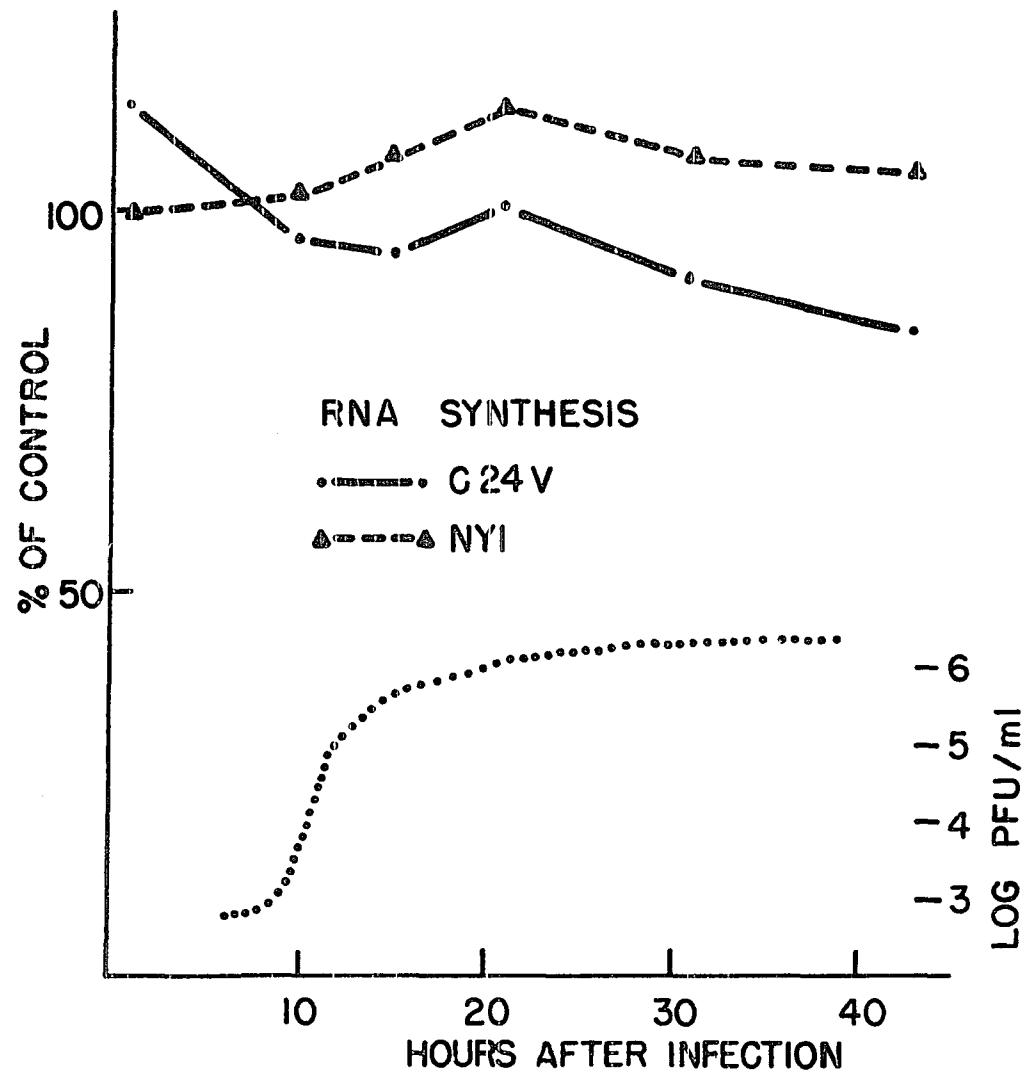


Figure 7. Total protein synthesis in cultures infected with C24V or NY1 virus. Triplicate samples were measured at the designated times. A growth curve for C24V virus is plotted as a reference. No significant differences were detected between viruses. The m.o.i. was 2.2 for both viruses

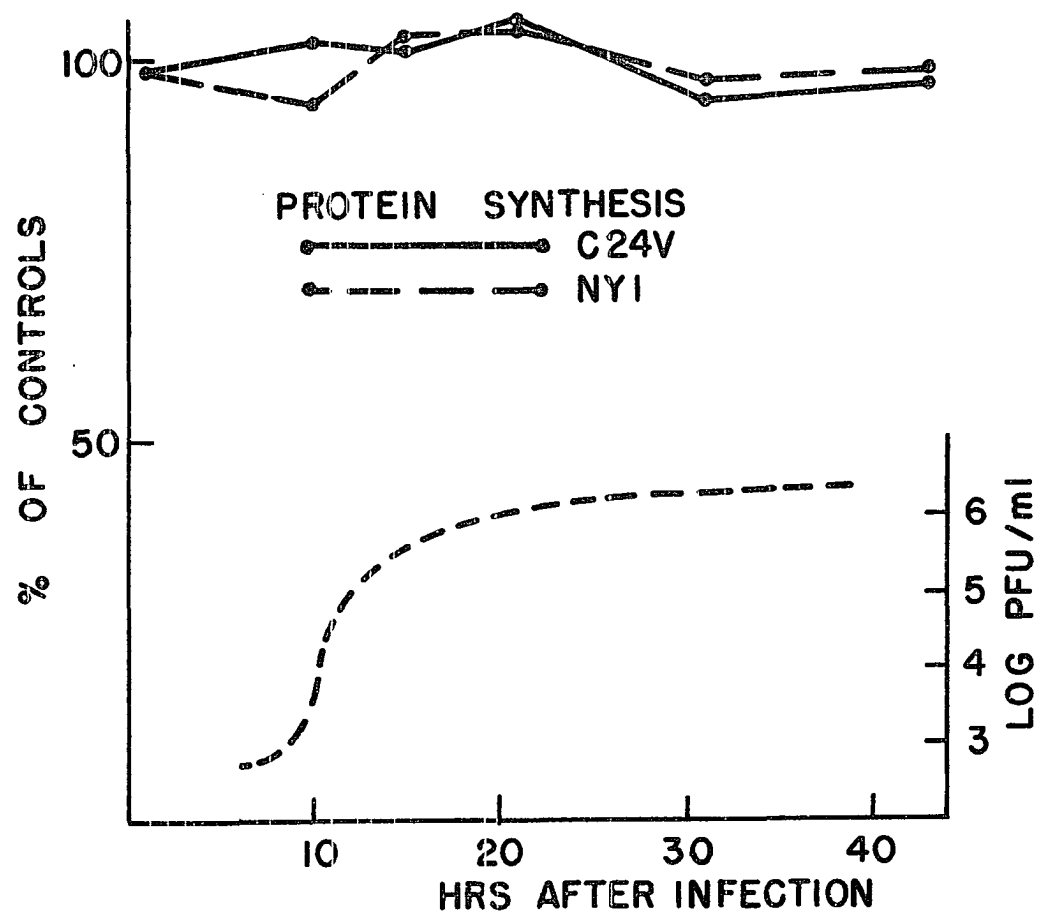


Figure 8. Leakage of C^{14} -labeled cellular proteins into the cell-free culture medium measured as disintegrations per minute of C^{14} in samples from duplicate cultures. Significant differences ($p < 0.01$) were detected between the effect caused by both viruses at 30 and 37 hours after infection. The m.o.i. was 1.3 for both viruses

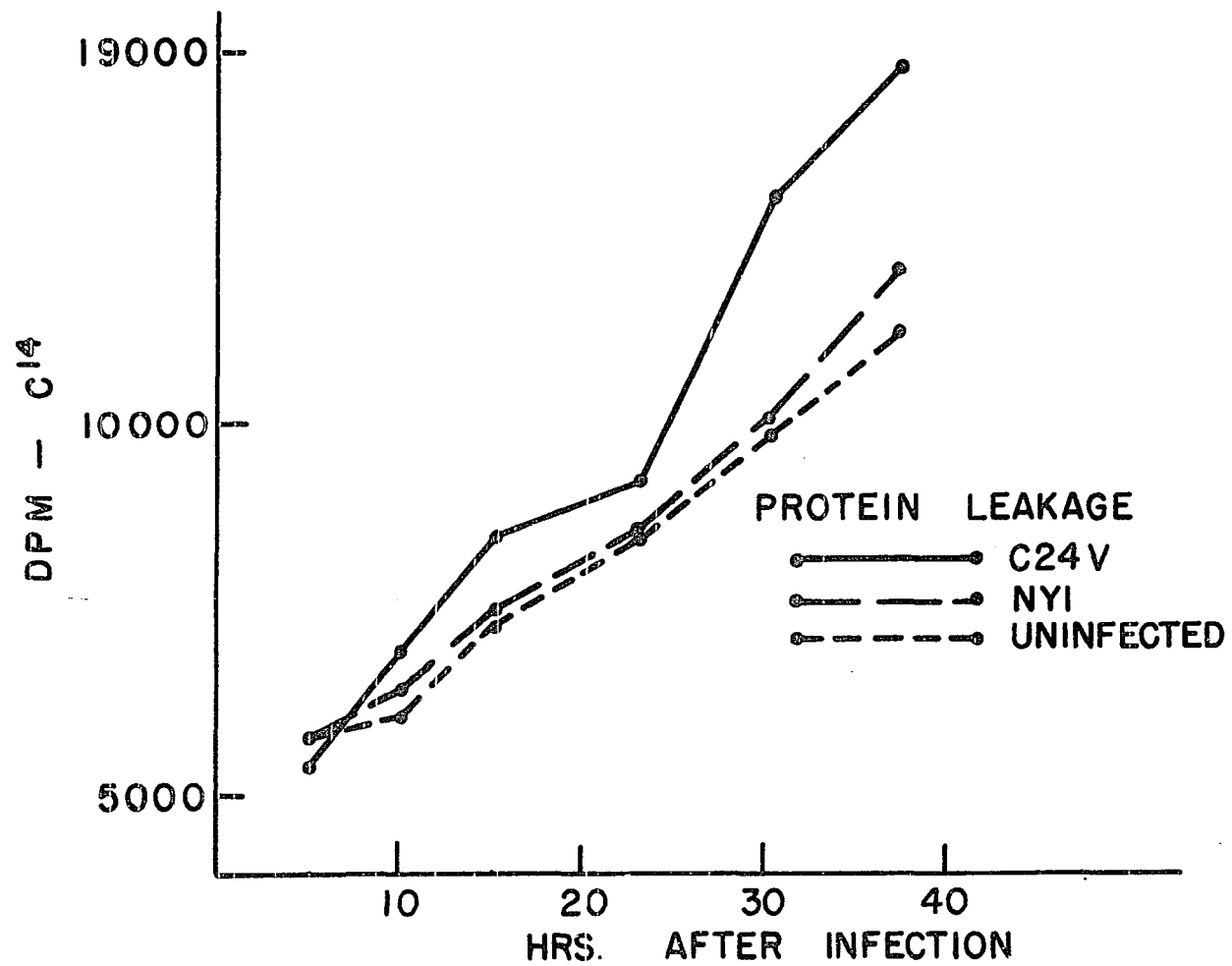
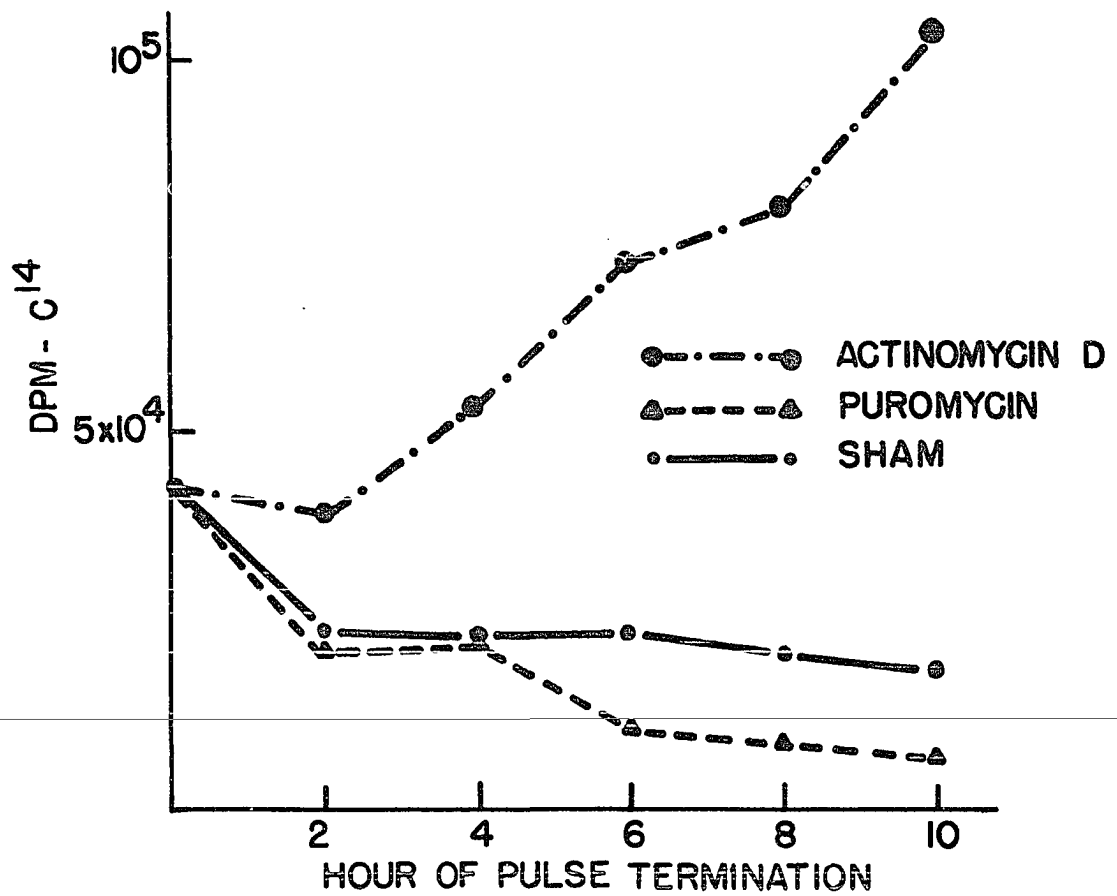


Figure 9. Inhibition of protein leakage caused by C24V virus infection at m.o.i. = 3.5. Pulses were given from 1 to 2, 2 to 4, 4 to 6, 6 to 8 and 8 to 10 hours after infection. Leakage of proteins was allowed to proceed from the time of pulse termination (values on the abscissa) to 30 hours. Leakage was measured as disintegrations per minute of C^{14} -labeled extracellular protein. Significant differences ($p < 0.01$) were noted after puromycin pulses from 4 to 6, 6 to 8 and 8 to 10 hours and after all actinomycin-D pulses



DISCUSSION

The infectivity of NY1 virus preparations was demonstrated by the production of interference against superinfection with C24V virus in primary bovine testicle cell cultures. Various techniques, including filtration through 25 nm filters, were used in attempts to separate infectivity from interfering activity. Filtration was not a satisfactory procedure since both the NY1 and C24V viruses were able to pass the filters (Table 2). These results are in contrast to those reported by many authors (52, 26, 47, 69, 23, 54, 49, 94, 32), with only a report by Fernelius (30) in agreement. The latter reported that in a few instances, BVD virus infectivity passed through 25 nm filters. It is interesting to note that some investigators (69, 23, 54, 49) reported that BVD and hog cholera viruses had electron-dense internal cores of 24 to 28 nm in diameter. Particles of this size would be expected to pass through a 25 nm filter.

The 25 nm filtrable infectivity of C24V virus detected in the present study appeared to differ from the intact virus particle in its sensitivity to trypsin, but was equally sensitive to acid treatment or ribonuclease digestion (Table 3). It is possible that the external viral proteins of this infectious component are not protected by the same associated structures such as lipids that are present in intact virus particles. The

observation of Fernelius and Velicer (33) that infectivity could be separated into two peaks by gel filtration on agarose columns is also evidence for the heterogeneous nature of infectious particles of BVD virus. However, this heterogeneity could not be resolved by density gradient centrifugation since a band of infectivity with a buoyant density of $1.15 \text{ g} \times \text{cm}^{-3}$ was observed (31).

The available data does not indicate whether these 25 nm filtrable infectious components are actually immature forms of the virus or are the product of degradation of 40 nm virus particles. The possibility that these filtrable components were due to a contaminating virus cannot be excluded. However, this is very unlikely since plaques produced by the 25 nm filtrable component of C24V virus were indistinguishable from those produced by the intact virus particle.

Aside from the question of the origin of the 25 nm filtrable infectivity, the role that might be played by this filtrable infectivity in the infection of cells and animals by BVD virus can only be hypothesized. There is a possibility that a 25 nm infectious particle could be carried further by air currents and reach entrance sites in susceptible animals with greater ease. But the fact that this filtrable fraction is more sensitive to the action of proteases than the intact virus would probably assign to it a lesser role in the maintenance of the virus in nature.

Gel filtration techniques proved useful in the separation of infectivity from interfering activity. When sham or NY1 virus materials were filtered through P60 columns, interfering activity was recovered in two separate fractions. One of these fractions eluted following the excluded fractions from a P30 column (Figure 2). Since this type of gel excludes globular proteins of a molecular weight of more than 30,000 it was concluded that the molecular weight of this fraction was 30,000 or less. It was assumed that this small molecular weight component with interfering activity was interferon produced by the primary bovine testicle cells since it was separable from the virus particles and its size corresponded well with that reported for bovine interferons. Furthermore, this material retained its activity even after being subjected to pH 2 dialysis.

Sham, NY1 and C24V fractions recovered in the excluded fraction of P60 and P150 columns also had interfering activity (Figure 2 and Table 7). The molecular weight of this interfering fraction was deduced to be larger than 150,000 since these columns exclude globular proteins possessing molecular weights of >60,000 and >150,000 respectively. Ultracentrifugation at 120,000 G for 20 hours sedimented this interfering activity (Table 6) which could mean that the molecular weight of the material was significantly higher than 150,000. The interfering activity in this fraction was active on

homologous (C24V) and heterologous (VSV) viruses and could have been due to the presence of an interferon inducer, such as bacterial endotoxins, in the virus and sham preparations. Alternatively, the activity could have been caused by a large molecular weight interferon. No reports on bovine interferons of this molecular weight were found in the literature, but the detection of rabbit interferons of >134,000 (88) and human interferons of 160,000 (37) in molecular weight support the possibility that bovine cells could produce interferons of large size.

A thorough inspection was made of the procedure used to obtain virus and sham preparations, in an attempt to determine if interferon-inducing substances were being introduced into these materials. It was found that Gram negative bacteria with the cultural characteristics of Pseudomonas sp. were growing on the walls of the plastic carboys used to store glass-distilled water for tissue culture procedures. These bacteria could have been the source of endotoxins for the tissue culture media, since the routine filtration techniques that rendered these media bacteriologically sterile would not have removed endotoxins. It is significant that when bacterial growth was prevented the sham and virus lots produced did not contain pH 2 resistant interfering activity for heterologous (VSV) viruses.

When C24V preparations were filtered through P60 columns, interfering activity was detected in three separable fractions. The first fraction recovered eluted with the void volume of the column. It was assumed that this material was related to the large component with interfering activity ($>150,000$ in molecular weight) seen in sham and NY1 lots. As discussed before, this activity could have been due to the presence of an interferon or an interferon inducer, such as bacterial endotoxin. The next two fractions recovered contained interfering activity which could not be related to activity obtained from similar gel filtration experiments with NY1 or sham preparations (Figure 1). No data is available to identify the nature of these interfering components.

The NY1 virus preparations contained a specific interfering activity that was not present in sham preparations. It was eluted as an unretained fraction from P150 columns which would indicate that its molecular weight was $>150,000$. This specific activity interfered with the homologous C24V virus but had no effect on VSV (Table 7). In addition, fractions of NY1 preparations that excluded from P150 columns contained the interferon or interferon inducer of $>150,000$ in molecular weight.

When NY1 virus was purified by ultrafiltration on XM50 membranes, materials of molecular weight lower than 50,000 were removed. All of the detectable interfering activity for

C24V could be removed from these preparations by ultracentrifugation at 120,000 G for 20 hours (Table 6). This centrifugal force also sedimented NY1 virus infectivity. Since ultracentrifugation removed the infectivity and the activity which specifically interfered with C24V virus, it is quite possible that both properties are associated with the virus particles. Further evidence that this was the case was the fact that only those cell cultures that were infected with NY1 virus became resistant to the challenge C24V virus. Cells infected with NY1 virus exhibited interference even after repeated washings, suggesting that the interference was due to an intracellular phenomenon or to the blocking of receptors at the cell surface. Experiments were performed to differentiate between these two alternative explanations but no conclusive data were obtained. In order to prove that interference between two viruses is mediated by the blocking of cellular receptors, a sufficiently high multiplicity of infection of the interfering virus must be used. Under these conditions a reduction in the rate of adsorption of the challenge virus is observed with respect to uninterfered cultures. The titers of NY1 virus obtained routinely, were not high enough to satisfactorily perform this test. To determine which of the various intracellular mechanisms of interference might be involved in the observed interference between two viruses, it

is necessary to use specific mutants which have not been obtained for NY1 virus to date.

The infectivity from NY1 virus preparations was eliminated by dialysis to pH 2. Preparations obtained with this technique contained interfering activity that was not retained by 25 nm filters or sedimented by centrifugation at 100,000 G for 1 hour (Table 4). This interfering activity was also demonstrated with C24V virus and VSV but not with IBR or BE and thus was due to interferon (Table 5). Since the small (30,000) molecular weight interferon was resistant to pH 2 it was assumed that at least part of the activity detectable in pH 2 dialysates of NY1 virus was due to this interferon. No experiments were performed to determine whether the large (>150,000) interfering component was resistant to pH 2 dialysis.

The sensitivity to interferon demonstrated by C24V virus was similar to that seen with VSV virus, a virus that is considered very sensitive to this inhibitor. This observation is in agreement with that of Diderholm and Dinter (25) who reported that C24V virus was sensitive to interferon.

Interference was also produced by NY1 virus irradiated with ultraviolet light (Table 6). It was not determined whether this interference was mediated by interferon or was produced by the same mechanisms as that expressed by unirradiated virus. Burke and Isaacs (18) have reported that

ultraviolet-irradiated influenza virus was a better interferon inducer than unirradiated virus.

It can be concluded, in agreement with Diderholm and Dinter (25), that interferon does not play a role in the establishment of interference between noncytopathogenic and cytopathogenic BVD strains. Contradictory reports by Kniazeff and McClain (62) and Gratzek (45) indicate that there was interference between noncytopathogenic BVD strains and VSV. It is possible that the results obtained by these authors were due to stimulation of interferon production by toxic bacterial products present in the water or animal serums used for cell cultures, even after sterilization by moist heat or filtration (99, 3).

In the present study, toxic bacterial products stimulated interferon production even in the presence of replicating NY1 virus. In contrast, Diderholm and Dinter (25) reported that a noncytopathogenic BVD strain suppressed the stimulation of interferon production by a viral inducer. This apparent contradiction may be due to the difference in interferon inducers.

In reviewing the subject of interferon synthesis by cells exposed to non-viral inducers, Ho et al. (53) pointed out that non-viral inducers will give interferon responses much sooner than viral inducers. Finkelstein et al. (36) reported that the interferon response elicited by non-viral inducers is more resistant to puromycin and actinomycin D than the one elicited by viral inducers. These latter authors have suggested that

non-viral inducers may release preformed interferon while viral inducers have to elicit "de novo" synthesis of interferon, a process that is blocked by actinomycin D and puromycin. In the present study, NY1 virus infection appeared to influence the interferon response in a similar manner to these drugs.

On the other hand, infection with NY1 virus did not result in detectable alterations of cell macromolecular metabolism (Figures 5, 6 and 7) as are produced by puromycin and actinomycin D. It is possible that NY1 virus specifically inhibited one of the steps required for the induction of interferon production by viral inducers. This step would not be required by non-viral inducers. Further studies with NY1 virus may provide useful information on the mechanisms of interferon induction.

It was of interest to note that gel filtration not only separated the interfering activity into distinct peaks but that certain large molecular weight fractions eluted from these columns also had enhancing activity on the C24V challenge (Figure 1). This enhancing activity was produced only in virus-infected cells since it was not detected in sham preparations.

The enhancing activity in NY1 fractions could have been due to products synthesized by this virus which would have a complementation effect on the challenge C24V virus. A very pronounced enhancing activity was seen in some fractions obtained from C24V preparations. This could be explained by

the fact that these 25 nm filtered materials contained some infectious C24V virus which produced plaques that were counted as due to the challenge virus.

The NY1 and C24V strains were remarkably similar in their kinetics of intracellular replication. Both released after an intracellular phase of 10 to 11 hours. No evidence of intracellular accumulation of infectivity was detected, as is compatible with the model of a virus that matures at the cell surface. The observed length of the growth cycle corresponds well with data reported previously for the cytopathogenic C24V strain (44, 45). Contrary to these findings, intracellular accumulation of virus was not detected.

A high m.o.i. of partially purified virus did not interfere with normal virus replication (Table 8). The yield of virus from cells infected with a m.o.i. of 1.3 (which would infect 75% of the cells) was one half of that obtained from cells infected with a m.o.i. of 90 (which would infect 100% of the cells). This multiplicity-dependent increase in virus yield was similar to the one seen with influenza virus by White and Cheyne (104) and is presumably due to the fact that some of the early viral proteins may not be produced in sufficient quantity to enable the rapid progress of replication. At high m.o.i., each cell is infected with more than one virus particle and no shortages of viral products occur. This phenomenon may

be related to the enhancement of C24V challenge seen with some fractions of C24V and NY1 virus (Figure 1).

The growth of C24V virus was markedly inhibited by actinomycin D during the first 3 hours of replication. Levels of 0.1 $\mu\text{g/ml}$ of the drug were able to exhibit this inhibition. This level of drug was considered "low" by Reich (77), so that C24V virus would have been classified as actinomycin D sensitive in his scheme. Aynaud (5) reported that the growth of hog cholera virus was resistant to actinomycin D at levels of 0.05 and 0.5 $\mu\text{g/ml}$ when used in pig kidney cells. It would be surprising if this difference in sensitivity to actinomycin D between hog cholera and BVD virus was due to a major difference in the replicative mechanism of these viruses, such as the need for cellular DNA synthesis during a part of the replicative cycle. Since hog cholera and BVD viruses are similar in so many respects it would be more logical to assume that the sensitivity to actinomycin D observed with C24V virus in primary bovine testicle cells is a characteristic of this particular virus-cell system. It is possible that certain strains of BVD virus could be resistant to the drug as could the C24V strain when grown in other host cells. Variations in sensitivity to actinomycin D among poliovirus strains have been reported by Schaffer and Gordon (84). Cooper (20) reported that the actinomycin D resistance of poliovirus strains was markedly influenced by the cultural conditions imposed on the host cells.

Autoradiographic studies indicated that cells infected with NY1 or C24V viruses had increased rates of nuclear RNA synthesis. These effects were marked at 14 hours but were noticeable at 8 hours even when 0.1 µg/ml of actinomycin D was added at 3 hours after infection. No significant changes were observed in the rates of cytoplasmic RNA synthesis of infected or uninfected cells. On the basis of these results, it is possible that NY1 and C24V viruses replicated their genomes in the nucleus of infected cells. Intranuclear replication of viral genomes is seen in infections with myxoviruses and paramyxoviruses. In contrast, arboviruses replicate their genomes in the cytoplasm. An intranuclear site of replication implies the possibility that the virus could interact with cell DNA and induce a state of malignancy in the host cell.

Skehel and Burke (87) observed that viral RNA polymerase activity was detectable in the cytoplasm and possibly the nucleus of infected cell homogenates. These results conflict with the autoradiographic experiments by Scholtissek et al.

(82) which demonstrated virus-directed synthesis only in the nucleus of cells. It is not clear at present why there is a discrepancy when measuring the same phenomenon with two different methods. Further experiments using these two techniques are needed to conclusively determine the intracellular site at which BVD viruses replicate their genome.

The localization of viral antigens with fluorescent antibody techniques can give information on the intracellular site of viral replication. The site at which antigens are detected may not be related to the one at which virus-induced protein synthesis occurs. Instead, it represents the location at which these viral proteins aggregate. Mengeling et al. (70) has reported that BVD and hog cholera viruses produce viral antigens in the cytoplasm of infected cells. Reports by Solorzano (90) and Fernelius (34) suggested that during the early phase of virus replication a faint nuclear fluorescence was apparent followed by the distinct cytoplasmic fluorescence that is typical of the later phases of replication with these viruses. The early nuclear fluorescence reported by these authors could be due to the aggregation of viral antigens in the nucleus under the direction of viral genomes that replicate at that site.

A distinct difference in the ability to inhibit cellular synthetic processes was noted between the cytopathogenic C24V and the noncytopathogenic NY1 strain. The C24V strain significantly inhibited cellular DNA synthesis at 43 hours after infection. This effect was followed by a corresponding inhibition of total RNA synthesis but protein synthesis remained unchanged during the period of observation. In contrast, cells infected with NY1 virus had no alterations of DNA, RNA or protein synthesis.

These patterns of inhibition of cellular metabolism can best be interpreted by assuming that the virus has a "late" effect, of either a direct or indirect nature, on the DNA of the cell and that this causes eventual inhibition of RNA synthesis. The measurements of RNA and protein synthesis are more unreliable as indicators of alterations of cellular metabolism because they are the result of measurements that combine both virus and cell-directed synthesis. These cellular alterations are much less dramatic than those typical of infections with mengovirus (38) or Newcastle disease virus (102).

Virus induced protein leakage can be measured by direct protein determinations of supernatant fluids or by determinations of the presence of lysosomal enzymes in the supernatant fluids. In this study, leakage of proteins was measured by determinations of the presence of radioactive cellular protein in the supernatant fluids. This eliminated the possibility of measuring virus-synthesized proteins in these supernatants. Bovine testicle cells normally leaked proteins into the supernatant fluids (Figure 8), reducing the sensitivity of the technique.

Marked differences in the ability to induce increased leakage of cellular proteins were noted between C24V and NY1 viruses. No alterations were caused by NY1 virus but C24V virus induced significantly increased levels of cellular protein leakage at 30 hours after infection (Figure 8). This

was a "late" effect, occurring after virus release but much earlier than the inhibition of cellular DNA synthesis caused by this virus. The fact that virus-induced protein leakage preceded the inhibitory action on cellular metabolism would indicate that this earlier effect is more directly associated to the lethal action of the virus on cells (Figure 4). Since NY1 virus did not induce protein leakage or inhibit cellular metabolism it can be concluded that these two effects contributed to the lethal action on the cells. The sequence of appearance of these cellular alterations could mean that the inhibition of cellular DNA synthesis was caused by this previous leakage of protein.

Cells infected with C24V virus had morphological alterations at 19 hours after infection (Figure 4). The leakage of cellular proteins from C24V-infected cells was slightly increased as early as 15 hours after infection (Figure 8) but only became significant at 30 hours after infection, when marked CPE was evident. It is likely that this delay in the appearance of protein leakage was only due to a lack of sensitivity in the detection of virus-specific leakage.

Protein synthesis was required shortly after 4 hours to enable the C24V virus to induce increased leakage of cellular proteins. Pulses of puromycin (50 $\mu\text{g}/\text{ml}$) that reversibly inhibited all protein synthesis caused a significant reduction in the leakage of cellular proteins from virus infected cells.

These results are strong evidence for the possibility that the C24V virus was producing a cytotoxic protein that damaged cellular lysosomes. This effect would then result in the detectable increases of cell protein leakage. Since puromycin was only effective in blocking this viral action beginning at 4 hours after infection, the protein which caused this effect must have been a "late" viral protein. This observation is in agreement with those of Amako and Dales (4) and Bubel (13) who showed that mengovirus produced a "late" protein capable of damaging cellular lysosomes beginning at 2.5 hours after infection. Kamiya et al. (61) also reported that a "late" protein of pseudorabies virus induced cell protein leakage. This "late" viral protein was produced beginning at 4 hours after infection.

Even though a protein capable of disrupting lysosomes is present in the cytoplasm of mengovirus-infected cells (97), no evidence has been presented to unequivocally demonstrate that this protein is directly produced by the virus genome. The possibility still exists that the virus genome interacts in some fashion with the cell DNA and causes it to direct the synthesis of a lysosome disrupting protein. When pulses of actinomycin D were used at different intervals after infection, the resultant leakage of cellular proteins was observed to increase the later the pulse was given. This was paradoxical, since later pulses meant that after removal of the drug pulse,

the cells had less time to release proteins than in the case of cultures pulsed earlier. It is possible that a cellular function was active in protecting the cell against viral injury during the infectious process. Pulses with actinomycin D, which binds irreversibly to DNA, could have rendered this cellular function impotent. This function presumably became more important later in the infectious cycle to counteract the increasingly lethal effects of the virus on lysosomes. The effect of pulses with actinomycin D produced such a marked leakage of proteins that the hypothesis of the existence of a cellular gene carrying information to disrupt lysosomes could not be tested by this method.

The NY1 and C24V strains of BVD virus were isolated from clinical cases of BVD infection in cattle (8, 42). Clinical syndromes in each one of these isolations were very similar. Nevertheless, these strains differ significantly when studied in cell cultures.

Amako and Dales (4) have presented data of experimental comparisons between a large and a small plaque variant of mengovirus. They observed that the large plaque variant was more virulent for mice and produced a larger amount of cytotoxic protein than the small plaque variant.

In the present study, the difference in cytopathogenicity between the NY1 and C24V strains was striking. The NY1 strain produces no cellular alterations while the C24V virus produces

late but marked cytopathology. In contrast to the observations of Amako and Dales, no clinical differences have been noticeable between these two strains. This discrepancy can be partially explained by the fact that the mengovirus strains were causing infection with rapid destruction of non-regenerating nervous tissue while in the case of BVD the infection produces a slower destruction of lymphoid tissue which can be regenerated. Fenner (29) has proposed that certain immunological reactions, such as delayed hypersensitivity, might play an important role in the cellular damage seen in animals during infections with viruses. The alteration of cellular plasma membranes by a virus could lead to the triggering of "graft rejection" reactions causing the destruction of these cells. This could be the reason why a noncytopathogenic virus, such as NY1 can produce symptoms, lesions and even death in animals. The fact that no intracellular accumulations of virus were observed with these strains of BVD is an indication that these viruses may mature at the cell surface and therefore necessarily alter the antigenicity of the cellular plasma membrane. The observations of Fernelius and Packer (35), that anticellular antibodies neutralized some strains of BVD virus, also lend support to this supposition.

Another explanation that cannot be excluded is that the NY1 virus is only noncytopathogenic in certain cell cultures

but is fully capable of lethal infections in many of the tissues of live animals. Much work will have to be done before one of these alternative hypotheses can be accepted for the BVD model. Such work would provide considerable insight and valuable information on the pathogenesis of BVD infections.

SUMMARY

The noncytopathogenic NY1 and cytopathogenic C24V strains were characterized as BVD viruses by chloroform sensitivity, nucleic acid type and virus neutralization. A fraction of the infectivity of these two strains passed through 25 nm filters. The 25 nm filtrable infectivity of C24V virus was more sensitive to trypsin digestion than the intact virus particle, but both infectious particles were sensitive to pH 2 and resistant to ribonuclease digestion.

Lots of NY1 virus, C24V virus and sham preparations contained materials which were separable from infectivity, resistant to pH 2 and had interfering activity against C24V and VSV viruses. Their molecular weights were approximately 30,000 and >150,000, as determined by gel filtration experiments. These properties identified the small molecular component as interferon and the large component as either an interferon or an interferon inducer. Interferon was removed from virus and sham preparations when the water used for tissue culture procedures was freed of toxic bacterial products.

A specific interference was detected only in NY1 virus lots. This material was filtrable through 25 nm filters, had a molecular weight of >150,000, was sedimented by ultracentrifugation, was destroyed by dialysis to pH 2, and was active only against the homologous C24V strain. This specific

interfering activity seemed to reside in the NY1 virus particle. It was concluded that the interference between noncytopathogenic and cytopathogenic BVD strains was not due to interferon but was mediated by the noncytopathogenic virus itself.

Certain column fractions from NY1 virus lots had an enhancing effect on the challenge C24V virus. This effect was possibly due to a complementation of the challenge virus by NY1 virus products.

After a growth cycle of 10 to 11 hours, NY1 and C24V viruses released from cells without any previous intracellular accumulation of infectious particles. These results are compatible with the model of a virus that matures at the cell surface.

High multiplicities of infection of C24V virus had no interfering effect; rather, they elicited a multiplicity-dependent increase in virus yield.

Autoradiographic evidence was obtained for an increase of nuclear RNA synthesis in cells infected with either virus. This increased RNA synthesis may be due to the replication of viral genomes in the nucleus. An actinomycin D sensitive phase was detected during the first 3 hours of replication of C24V virus.

The C24V strain gave initial CPE at 19 hours, appreciable leakage of C^{14} -labeled cellular proteins at 30 hours and

marked inhibition of cellular DNA synthesis at 43 hours. This was followed later by inhibition of RNA synthesis. The NY1 strain failed to cause these changes in cell cultures. It was assumed that the C24V strain produced cytopathology by altering lysosomes, which resulted in the leakage of cellular proteins and later, in inhibitions of cell macromolecular metabolism. Since protein synthesis was required 4 hours after infection with C24V in order to cause increased cell protein leakage, it was concluded that a "late" viral protein was responsible for this lethal effect.

BIBLIOGRAPHY

1. Adamik, E. R. Laboratory procedures for tritium autoradiographs. Orangeburg, New York, Schwarz BioResearch, Inc. ca. 1960.
2. Allison, A. C. and L. Mallucci. Histochemical studies of lysosomes and lysosomal enzymes in virus-infected cell cultures. *Journal of Experimental Medicine* 121: 463-474. 1965.
3. Allison, M. J., M. R. Escobar, H. D. Dalton and B. Wright. Use of cell culture to detect bacterial products (Abstract). *Bacteriological Proceedings* 1969: A46. 1969.
4. Amako, K. and S. Dales. Cytopathology of mengovirus infection. I. Relationship between cellular disintegration and virulence. *Virology* 32: 184-200. 1967.
5. Aynaud, J. M. Étude de la multiplication in vitro d'un clone du virus de la peste porcine. *Recherches Vétérinaires* 1: 25-36. 1968.
6. Bablanian, R., H. J. Eggers and I. Tamm. Studies on the mechanism of poliovirus-induced cell damage. I. The relation between poliovirus-induced metabolic and morphological alterations in cultured cells. *Virology* 26: 110-113. 1965.
7. Bablanian, R., H. J. Eggers and I. Tamm. Studies on the mechanism of poliovirus-induced cell damage. II. The relation between poliovirus growth and virus-induced morphological changes in cells. *Virology* 26: 114-121. 1965.
8. Baker, J. A., C. J. York, J. H. Gillespie and G. B. Mitchell. Virus diarrhea in cattle. *American Journal of Veterinary Research* 15: 525-531. 1954.
9. Balandin, I. G. and L. N. Kastrikin. Studies of an inhibitor of RNA polymerase appearing in virus-infected cells. I. Effects of the inhibitor on the activity of RNA polymerase from *E. Coli* in vitro. *Acta Virologica* 11: 403-406. 1967.
10. Baluda, M. A. Loss of viral receptors in homologous interference by ultraviolet-irradiated Newcastle disease virus. *Virology* 7: 315-327. 1959.

11. Barry, R. D. The effects of actinomycin D and ultraviolet irradiation on the production of fowl plague virus. *Virology* 24: 563-569. 1964.
12. Ben-Ishai, Z., N. Goldblum and Y. Becker. The intracellular site and sequence of sindbis virus (SV) replication. *Journal of General Virology* 2: 365-375. 1968.
13. Bubel, H. C. Protein leakage from mengovirus-infected cells. *Society for Experimental Biology and Medicine Proceedings* 125: 783-786. 1967.
14. Buck, C., G. Granger, M. Taylor and J. Holland. Efficient, inefficient and abortive infection of different mammalian cells by small RNA viruses. *Virology* 33: 36-46. 1967.
15. Bukrinskaya, A. G. and V. M. Zhdanov. Shortening by actinomycin D of latent period of multiplication of Sendai virus. *Nature* 200: 920-921. 1963.
16. Bukrinskaya, A. G. Resistance of synthesis of influenza virus ribonucleic acid to the action of actinomycin D. *Nature* 204: 205-206. 1964.
17. Bukrinskaya, A. G., O. Burducea and G. K. Vorkunova. The intracellular site of RNA synthesis of Newcastle disease virus. *Society for Experimental Biology and Medicine Proceedings* 123: 236-238. 1966.
18. Burke, D. C. and A. Isaacs. Further studies on interferon. *British Journal of Experimental Pathology* 39: 78-84. 1958.
19. Bürki, F. Eigenschaften des virus der equinen arteritis. *Pathologia et Microbiologia* 28: 939-949. 1965.
20. Cooper, P. D. The inhibition of poliovirus growth by actinomycin D and the prevention of the inhibition by pretreatment of the cells with serum or insulin. *Virology* 28: 663-678. 1966.
21. Cords, C. E. and J. J. Holland. Interference between enteroviruses and conditions effecting its reversal. *Virology* 22: 226-234. 1964.
22. Crowell, R. L. Specific cell surface alteration by enteroviruses as reflected by viral-attachment interference. *Journal of Bacteriology* 91: 198-204. 1966.

23. Cunliffe, H. The purification and concentration of hog cholera virus. Unpublished M.S. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology. 1967.
24. Darbyshire, J. H. The gel diffusion technique in studies of viruses of veterinary importance. II. Applications to swine fever and mucosal disease. *Veterinary Bulletin* 35: 71-76. 1965.
25. Diderholm, H. and Z. Dinter. Interference between strains of bovine virus diarrhea virus and their capacity to suppress interferon of a heterologous virus. *Society for Experimental Biology and Medicine Proceedings* 121: 976-980. 1966.
26. Dinter, Z. Relationship between bovine virus diarrhea virus and hog cholera virus. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* 188: 475-486. 1963.
27. Dubes, G. R. and H. Rouhandeh. Interference between poliovirus ribonucleic acids. *Archiv für die Gesamte Virusforschung* 14: 87-98. 1963.
28. Fantes, K. H. Purification, concentration and physico-chemical properties of interferons. In Finter, N. B., ed. *Frontiers of biology*. Vol. 2. Pp. 119-180. Philadelphia, Pennsylvania, W. B. Saunders Co. 1966.
29. Fenner, F. The biology of animal viruses. II. The pathogenesis and ecology of viral infections. New York, New York, Academic Press, Inc. 1968.
30. Fernelius, A. L. Characterization of bovine viral diarrhea viruses by their interactions with susceptible host cells. Unpublished Ph.D. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology. 1967.
31. Fernelius, A. L. Characterization of bovine viral diarrhea viruses. I. Determination of buoyant density. *Archiv für die Gesamte Virusforschung* 25: 211-218. 1968.
32. Fernelius, A. L. Characterization of bovine viral diarrhea viruses. II. Ultrafiltration properties of different strains after various treatments. *Archiv für die Gesamte Virusforschung* 25: 219-226. 1968.

33. Fernelius, A. L. and L. F. Velicer. Characterisation of bovine viral diarrhea viruses. III. Partial purification by agar gel filtration. Archiv für die Gesamte Virusforschung 25: 227-234. 1968.
34. Fernelius, A. L. Characterization of bovine viral diarrhea viruses. IV. Sequential development of BVD viral antigen in cell cultures studied by immunofluorescence. Archiv für die Gesamte Virusforschung. In press. 1969.
35. Fernelius, A. L. and R. A. Packer. Reactions of bovine diarrhea viruses with anticellular and antiviral sera (Abstract). Bacteriological Proceedings 1969: V20. 1969.
36. Finkelstein, M. S., G. H. Bausek and T. C. Merigan. Interferon inducers in vitro: difference in sensitivity to inhibitors of RNA and protein synthesis. Science 161: 465-467. 1968.
37. Fournier, F., E. Falcoff and C. Chany. Demonstration, mass production and characterization of a heavy molecular weight human interferon. Journal of Immunology 99: 1036-1041. 1967.
38. Franklin, R. M. and D. Baltimore. Patterns of macromolecular synthesis in normal and virus-infected mammalian cells. Cold Spring Harbor Symposia in Quantitative Biology 27: 175-195. 1962.
39. Friedmann, R. M. and J. A. Sonnabend. Inhibition of interferon action by p-fluorophenylalanine. Nature 203: 366-367. 1964.
40. Friedman, R. M. and I. K. Berezesky. Cytoplasmic fractions associated with Semliki Forest virus ribonucleic acid replication. Journal of Virology 1: 374-383. 1967.
41. Gauntt, C. J. and R. Z. Lockart. Destruction of L cells by mengovirus: use of interferon to study the mechanism. Journal of Virology 2: 567-575. 1968.
42. Gillespie, J. H., J. A. Baker and K. McEntee. A cytopathogenic strain of virus diarrhea virus. Cornell Veterinarian 50: 73-79. 1960.

43. Gillespie, J. H., S. H. Madin and N. B. Darby, Jr. Cellular resistance in tissue culture, induced by non-cytopathogenic strains, to a cytopathogenic strain of virus diarrhea virus of cattle. *Society for Experimental Biology and Medicine Proceedings* 110: 248-250. 1962.
44. Gillespie, J. H., S. H. Madin and N. B. Darby, Jr. Studies on virus diarrhea virus of cattle, with special reference to its growth in tissue culture and its ether susceptibility. *Cornell Veterinarian* 53: 276-282. 1963.
45. Gratzek, J. B. Characteristics of two bovine viral diarrhea agents. Unpublished Ph.D. thesis. Madison, Wisconsin, Library, University of Wisconsin. Xeroxed copy Order No. 62-1173. Ann Arbor, Michigan, University Microfilms, Inc. 1962.
46. Gutekunst, D. E. and W. A. Malmquist. Separation of a soluble antigen and infectious particles of bovine viral diarrhea viruses and their relationship to hog cholera. *Canadian Journal of Comparative Medicine and Veterinary Science* 27: 121-123. 1963.
47. Gutekunst, D. E. Characterization of a bovine viral diarrhea agent. Unpublished M.S. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology. 1964.
48. Gutekunst, D. E. and W. A. Malmquist. Soluble antigen of bovine viral diarrhea virus. I. Intracellular synthesis, separation and characterization. *Archiv für die Gesamte Virusforschung* 15: 159-168. 1965.
49. Hafez, S. M., K. Petzoldt and E. Reczko. Morphology of bovine viral diarrhea virus. *Acta Virologica* 12: 471-473. 1968.
50. Hallum, J. V., J. S. Youngner and W. R. Stinebring. Interferon activity associated with high molecular weight proteins in the circulation of mice injected with endotoxin or bacteria. *Virology* 27: 429-431. 1965.
51. Heller, E. Enhancement of Chikungunya virus replication and inhibition of interferon production by actinomycin D. *Virology* 21: 652-656. 1963.
52. Hermodsson, S. and Z. Dinter. Properties of bovine virus diarrhea virus. *Nature* 194: 893-894. 1962.

53. Ho, M., K. H. Fantes, D. C. Burke and N. B. Finter. Interferons or interferon-like inhibitors induced by non-viral substances. In Finter, N. B., ed. *Frontiers of biology*. Vol. 2. Pp. 181-201. Philadelphia, Pennsylvania, W. B. Saunders Co. 1966.
54. Horzinek, M., E. Reczko and K. Petzoldt. On the morphology of hog cholera virus. *Archiv für die Gesamte Virusforschung* 21: 475-478. 1967.
55. Huang, A. S. and R. R. Wagner. Defective T particles of vesicular stomatitis virus. II. Biologic role in homologous interference. *Virology* 30: 173-181. 1966.
56. Isaacs, A. and J. Lindenmann. Virus interference. I. The Interferon. *Royal Society of London Proceedings. Series B.* 147: 258-267. 1957.
57. Isaacs, A. and M. A. Westwood. Inhibition by interferon of the growth of vaccinia virus in the rabbit skin. *Lancet* 2: 324-325. 1959.
58. Isaacs, A., R. A. Cox and Z. Rotem. Foreign nucleic acids as the stimulus to make interferon. *Lancet* 2: 113-116. 1963.
59. Jacob, F. and A. Campbell. Sur le système de répression assurant l'immunité chez les bactéries lysogènes. *Academic Scientifique du Paris Comptes Rendus* 248: 3219-3221. 1959.
60. Jacob, F. and J. Monod. Genetic regulatory mechanisms in the synthesis of proteins. *Journal of Molecular Biology* 3: 318-356. 1961.
61. Kamiya, T., T. Ben-Porat and A. S. Kaplan. Control of certain aspects of the infective process by progeny viral DNA. *Virology* 26: 577-589. 1965.
62. Kniazeff, A. J. and M. McClain. Annual report to the NC-34 technical committee on mucosal diseases of cattle. 1959. Cited in Pritchard, W. R. The bovine viral-diarrhea-mucosal disease complex. *Advances in Veterinary Science* 8: 39-40. 1963.
63. Levine, A. J. and H. S. Ginsberg. Role of adenovirus structural proteins in the cessation of host-cell biosynthetic functions. *Journal of Virology* 2: 430-439. 1968.

64. Lindenmann, J., D. C. Burke and A. Isaacs. Studies on the production, mode of action and properties of interferon. *British Journal of Experimental Pathology* 38: 551-562. 1957.
65. Lockart, R. Z., Jr. The necessity for cellular RNA and protein synthesis for viral inhibition resulting from interferon. *Biochemical and Biophysical Research Communications* 15: 513-518. 1964.
66. Malmquist, W. A., A. L. Fernelius and D. E. Gutekunst. Interference of bovine viral diarrhea virus by hog cholera virus in swine kidney cell cultures. *American Journal of Veterinary Research* 26: 1316-1327. 1965.
67. Marcus, P. I. and J. M. Salb. Molecular basis of interferon action: inhibition of viral RNA translation. *Virology* 30: 502-516. 1966.
68. Marcus, P. I. and D. H. Carver. Intrinsic interference: a new type of viral interference. *Journal of Virology* 1: 334-343. 1967.
69. Mayr, A., P. A. Bachmann, B. E. Sheffy and G. Siegl. Electron optical and buoyant density studies of hog cholera virus. *Archiv für die Gesamte Virusforschung* 21: 113-119. 1967.
70. Mengeling, W. L., D. E. Gutekunst, A. L. Fernelius and E. C. Pirtle. Demonstration of an antigenic relationship between hog cholera and bovine viral diarrhea viruses by immunofluorescence. *Canadian Journal of Comparative Medicine and Veterinary Science* 27: 162-164. 1963.
71. Mengeling, W. L., E. C. Pirtle and J. P. Torrey. Identification of hog cholera viral antigen by immunofluorescence. *Canadian Journal of Comparative Medicine and Veterinary Science* 27: 249-252. 1963.
72. Moss, B. Inhibition of HeLa cell protein synthesis by the vaccinia virion. *Journal of Virology* 2: 1028-1037. 1968.
73. Perry, R. P., M. Errera, A. Hell and H. Dürwald. Kinetics of nucleoside incorporation into nuclear and cytoplasmic RNA. *Biophysical and Biochemical Cytology Journal* 11: 1-13. 1961.

74. Plagemann, P. G. W. and H. E. Swim. Replication of mengovirus. I. Effect on synthesis of macromolecules by host-cell. *Journal of Bacteriology* 91: 2317-2326. 1966.
75. Pons, M. Effect of actinomycin D on the replication of influenza virus and influenza virus RNA. *Virology* 33: 150-154. 1967.
76. Porterfield, J. S. Interference and interferon. In Harris, R. J. C., ed. *Techniques in experimental virology*. Pp. 309-313. New York, New York, Academic Press, Inc. 1964.
77. Reich, E. Biochemistry of actinomycins. *Cancer Research* 23: 1428-1441. 1963.
78. Robertson, A., A. S. Grieg, M. Appel, A. Girard, G. L. Bannister and P. Boulanger. Hog Cholera. IV. Detection of the virus in tissue culture preparations by the fluorescent antibody technique. *Canadian Journal of Comparative Medicine and Veterinary Science* 29: 234-241. 1965.
79. Robertson, H., R. E. Webster and N. D. Zinder. Bacteriophage coat protein as repressor. *Nature* 218: 533-536. 1968.
80. Roizman, B. Abortive infection of canine cells by herpes simplex virus. III. The interference of conditional lethal virus with an extended host range mutant. *Virology* 27: 113-117. 1965.
81. Salzman, N. B., R. Z. Lockhart and E. D. Sebring. Alterations in HeLa cell metabolism resulting from polio-virus infection. *Virology* 9: 244-259. 1959.
82. Scholtissek, C., R. Rott, P. Hausen, H. Hausen and W. Schäfer. Comparative studies of RNA and protein synthesis with a myxovirus and a small polyhedral virus. *Cold Spring Harbor Symposia in Quantitative Biology* 27: 245-257. 1962.
83. Scholtissek, C., H. Becht and R. Drzeniek. Biochemical studies on the cytopathic effect of influenza viruses. *Journal of General Virology* 1: 219-225. 1967.

84. Schaffer, F. L. and M. Gordon. Differential inhibitory effects of actinomycin D among strains of poliovirus. *Journal of Bacteriology* 91: 2309-2316. 1966.
85. Schlesinger, W. Interference between animal viruses. In Burnet, F. M. and W. M. Stanley, eds. *The viruses*. Vol. 3. Pp. 157-194. New York, New York, Academic Press, Inc. 1959.
86. Singh, K. V. Homologous Interference by an actively multiplying noncytopathic strain of bovine virus diarrhea-mucosal virus. *Applied Microbiology* 17: 476-479. 1969.
87. Skehel, J. J. and D. C. Burke. Ribonucleic acid synthesis in chick embryo cells infected with fowl-plague virus. *Journal of Virology* 3: 429-438. 1969.
88. Smith, T. J. and R. R. Wagner. Rabbit macrophage interferons. II. Some physiochemical properties and estimations of molecular weights. *Journal of Experimental Medicine* 125: 579-593. 1967.
89. Snedecor, G. W. *Statistical methods*. 5th ed. Ames, Iowa, Iowa State University Press. 1964.
90. Solorzano, R. F. A fluorescent antibody test for hog cholera. Unpublished Ph.D. thesis. University Park, Pennsylvania, Library, Pennsylvania State University. 1962.
91. Steck, F. T. and H. Rubin. The mechanism of interference between an avian leukosis virus and Rous sarcoma virus. I. Establishment of interference. *Virology* 29: 628-641. 1966.
92. Stinebring, W. R. and J. S. Youngner. Patterns of interferon appearance in mice injected with bacteria or bacterial endotoxin. *Nature* 204: 712. 1964.
93. Sydiskis, R. J. and B. Roizman. The disaggregation of host polyribosomes in productive and abortive infection with herpes simplex virus. *Virology* 32: 678-686. 1967.
94. Tanaka, Y., Y. Inaba, T. Omori and M. Matumoto. Bovine diarrhea virus. III. Physiochemical properties of bovine diarrhea virus. *Japanese Journal of Microbiology* 12: 201-210. 1968.

95. Taylor, J. Inhibition of interferon action by actinomycin. *Biochemical and Biophysical Research Communications* 14: 447-451. 1964.
96. Ter Meulen, V. and R. Love. Virological, immunochemical and cytochemical studies of four HeLa cell lines infected with two strains of influenza virus. *Journal of Virology* 1: 626-639. 1967.
97. Thacore, H. and D. A. Wolff. Activation of isolated lysosomes by poliovirus-infected cell extracts. *Nature* 218: 1063-1064. 1968.
98. Wagner, R. R. Viral interference. *Bacteriological Reviews* 24: 151-166. 1960.
99. Wagner, R. R. and T. J. Smith. On the apparent heterogeneity of rabbit interferons. In Wolstenholme, G. E. W. and M. O'Connor, eds. *CIBA Foundation Symposium: Interferon*. Pp. 95-106. Boston, Massachusetts, Little, Brown and Co. 1967.
100. Walker, D. L. In vitro cell-virus relationships resulting in cell death. *Annual Review of Microbiology* 14: 177-196. 1960.
101. Wecker, E. and A. Richter. Conditions for the replication of infectious viral RNA. *Cold Spring Harbor Symposia in Quantitative Biology* 27: 51-55. 1962.
102. Wheelock, E. F. and I. Tamm. Biochemical basis for alterations in structure and function of HeLa cells infected with Newcastle disease virus. *Journal of Experimental Medicine* 114: 617-632. 1961.
103. Wheelock, E. F. Intracellular site of Newcastle disease virus nucleic acid synthesis. *Society for Experimental Biology and Medicine Proceedings* 114: 56-60. 1963.
104. White, D. O. and I. M. Cheyne. Early events in the eclipse phase of influenza and parainfluenza virus infection. *Virology* 29: 49-59. 1966.
105. Wilner, B. I. A classification of the major groups of human and other animal viruses. 4th ed. Minneapolis, Minnesota, Burgess Publishing Co. 1969.

106. Zebrovitz, E. and A. Brown. Interference among group A arboviruses. *Journal of Virology* 2: 1283-1289. 1968.
107. Zuckerbraun, H. L. and P. I. Marcus. Induction of intrinsic interference by temperature sensitive (ts) mutants of sindbis virus (Abstract). *Bacteriological Proceedings* 1969: V125. 1969.

ACKNOWLEDGMENTS

The author wishes to thank Dr. M. L. Kaeberle for his encouragement and guidance throughout this project and particularly for his help in the preparation of this manuscript.

Thanks are also extended to Drs. H. W. Davey, D. J. Graves, R. A. Packer and P. A. Pattee for their valuable suggestions. The assistance of Dr. D. F. Cox with the statistical procedures employed is gratefully acknowledged.

Appreciation goes to the entire staff of the Department of Veterinary Microbiology for the services which helped to make this work possible. Special thanks also go to Dr. C. J. Mare for the use of the virology laboratory for much of this work.

Many thanks are given to my wife for the many hours spent in the typing of the preliminary draft of this manuscript, and for her continuing encouragement.

This work was supported by Training and General Research Support grants from the National Institutes of Health, USPHS.